Fimbriae of *Coprinus cinereus*, *Schizophyllum commune* and *Ustilago violacea*: Structural Aspects and a Role in Conjugation

by

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A Thesis
submitted to the Depatment of Biological Sciences
in partial fulfillment of the requirements
for the degree of
Master of Science

August 1990
Brock University
St. Catharines, Ontario

Abstract

Surface fibrils (fimbriae) have been observed on fungi from every major group. Fimbriae are thought to be involved in the following cell to cell interactions: conjugation, flocculation and adhesion. Several higher fungi exibit two other types of interactions: hyphal fusion (anastomosis) and clamp connection formation. As a prelude to examining the role of fimbriae in these processes, the fimbriae of two fungi that undergo these fusion events were examined.

Electron microscopy studies revealed that *Coprinus cinereus* and *Schizophyllum commune* are fimbriated. *C. cinereus* fimbriae were 5 nm in diameter and 0.5 to 20 μm in length. Fimbriae of *C. cinereus* oidia were more numerous and longer than those of the hyphal stage. *S. commune* fimbriae were also 5 nm in diameter, but were only 0.5 to 2 μm in length. There was an unequal distribution of fimbriae on the hyphal surfaces of *S. commune* . Fimbriae were sparsely distributed over the entire hyphal surface, with higher densities of fibrils present on the side growths of the hyphae found in the older sections of the mycelium.

Antiserum raised against *Ustilago violacea* fimbrial protein (AU) cross-reacted strongly with 37 and 39 kd *C. cinereus* mycelial proteins. In contrast, AU bound very weakly to 89 and 92 kd *S. commune* mycelial proteins. Since AU cross-reacted poorly with *S. commune* fimbrial proteins, it was impossible to further characterize the fimbriae of this species. The 37 and 39 kd *C. cinereus* proteins, were isolated by electroelution and were shown to be able to form fibrils the same diameter as oidial fimbriae. The 37 kd protein was shown to be composed of several proteins with isoelectric points ranging from pH 6.1 to 7.63. Furthermore,

the 37 kd protein was found to be multimeric, while the 39 kd protein was not. These results strongly suggested that the 37 kd protein is the structural fimbrial protein of *C. cinereus* .

Finally, a series of experiments were designed to determine whether fimbriae are required for conjugation in *U. violacea* . Conjugation was inhibited significantly with AU, but not with pre-immune serum or AU preincubated with purified fimbrial protein. Thus, it was concluded that fimbriae play a central role in mating in this organism.

Acknowledgements

I would like to thank Dr. Castle for his help and understanding. I am grateful to Dr. B. C. Lu and Dr. B. Ulrich for donating their cultures of *C. cinereus* and *S. commune*. I would also like to thank Dr. Day for graciously providing the antibodies used in this study. Dr. Day was also kind enough to let me work in his lab several times over the course of my thesis work. I really appreciated the help of Ron Smith, who worked with me to complete the EM portion of my thesis. Without him, it wouldn't have been possible.

All of the people who passed through the Castle/Carlone lab were helpful. I would especially like to thank those that offered diversions from the lab. Thanks, Bill and Scott, for frisbee golf, nerf basketball and stairwell baseball. Scott was also very helpful in assisting with my 2-D gels. Most of all, I would like to thank Mitzi for making my time here enjoyable. She also helped considerably in the preparation of my thesis. Finally, the confidence and support of my mother was invaluable.

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Introduction

Fungal fimbriae were first discovered on *Ustilago violacea* in the mid-1970's by Poon and Day (1974). In addition, many other fungi have been found to have fimbriae. However, relatively little work has focused on the function that fimbriae might perform. In *Ustilago* , they are thought to mediate sexual conjugation between mating haploid cells (Day et al., 1975) and in *Saccharomyces* their presence is necessary for flocculation to occur (Day and Poon, 1974). Most of the fungi studied to date are yeast-like; unicellular fungi are easier to examine for this character than filamentous fungi. Thus, the investigation of the putative role of fimbriae has focused on their function(s) in unicellular fungi. This is unfortunate because filamentous fungi perform some fascinating interactions between cells. Of these intercellular interactions, the fusion of hyphae is of particular interest. Hyphal fusions can be divided into three separate classes: 1) sexual fusions, 2) vegetative fusions and 3) parasitic fusions. The formation of clamp connections is one form of vegetative fusion. In the hyphal stages of most Basidiomycetes, nuclear fusion does not normally occur during vegetative growth. Instead, the haploid nuclei remain separate and divide co-ordinately in elongating hyphal tips. One nucleus divides in the longitudinal plane of the hypha. The other nucleus divides into a side growth which bypasses the newly formed septum and fuses with the sub-terminal cell.

Fusions between hyphae, a process known as "anastomosis", usually occur in the older sections of the mycelium (Buller, 1933). Buller hypothesized that this phenomenon is induced by a depletion of nutrients. The interconnections between hyphae permits the distribution of nutrients

between all cells in the mycelium. Thus, this allows each cell within the mycelium access to nutrients, which would lead to increased growth. Typically, this fusion event occurs between a growing hyphal tip and an opposing hyphal tip or peg present on another hypha. The occurrence of tip to tip fusions is remarkable. The signalling process must be exact to produce such a precise growth response; especially within a large network of intertwining hyphae where the number of competing signals would be tremendous. Intuitively, it seems unlikely that simple diffusion of chemical signals is responsible for initiation and directed growth of hyphae during anastomosis. Direct physical contact and exchange of information between hyphae constitute more plausible events during the early stages of this process. Surface structures such as fimbriae might mediate this physical contact. To initiate studies on the potential role of fimbriae in hyphal fusions, it is necessary to ascertain that fungi which undergo these processes are fimbriated. Furthermore, it is necessary to partially characterize the structure of fimbriae of these fungi in order to determine ways in which one could examine the role of fimbriae in these processes.

The aims of this study were to:

a) establish the presence of fimbriae on *Coprinus cinereus* and *Schizophyllum commune* , extensively studied fungi that undergo both hyphal anastomosis and the formation of clamp connections,

b) initiate studies of the fimbrial proteins of these fungi and their relationship to the fimbrial proteins of other fungi,

c) examine the possibility that conjugation in *U. violacea* could be inhibited with antibodies raised against its fimbrial protein and confirm the requirement of fimbriae for cell fusions in this fungus.

Literature Review

Bacterial Fimbriae and Pili

Surface fibrils were first observed on bacteria in the late 1940's (Anderson 1949; Houwink and van Iterson, 1950). Since then, they have been referred to as filaments, bristles, fimbriae and pili (Ottow, 1975). Unfortunately, there still remains a discrepancy as to the correct term that should be used. The terms "fimbriae", which in Latin means thread or fibre (Duguid et al., 1955) and "pili", which in Latin means hair or hair like structure (Brinton, 1959) have been used interchangeably. In this review, the term fimbriae will be restricted to non-flagellar surface fibrils not associated with the transfer of DNA, whereas the term pili will be used to describe those non-flagellar structures associated with bacterial DNA transfer. This terminology follows the recommendations of Ottow (1975).

Fimbriae were thought at first to be restricted only to gram negative bacteria. However, upon examining other species of prokaryotes, every order with the exception of Rickettsiales were found to have fimbriated members. Most of the work concerning fimbriae has centered around the Enterobacteriaceae. This family of bacteria is important since many of its members are involved in the pathogenesis of man. It has also been found to have the largest number of fimbriated species.

Several different types of fimbriae have been identified in the last 40 years. To complicate matters, many bacterial strains have been observed to express two or more types simultaneously (Morris et al., 1980; Levine et al., 1984). Brinton (1965) devised a scheme that differentiated six types (types I-V and F) of fimbriae based upon their ultrastructure and

biochemistry. Duguid (1968) distinguished seven types (types 1-6 and F) based upon their agglutination properties and physical appearance. However, both of these plans were developed based on information gathered largely from *Escherichia coli.* Unfortunately, as more information was obtained from this and other species, it became apparent that neither plan was adequate to include all of the fimbriae types. Today, only the type 1 and F class of Duguid's scheme are in general use. Other fimbrial types have been distinguished based on their antigenicity. These include the K88, K99, CFA1, CS2 and CS3 fimbriae. There is no widely used, general scheme for their designation. However, Orskov and Orskov (1983), have recently developed a scheme which designates fimbrial types with an "F" followed by a code. For example, the F41 and the F7 and F12 series of fimbrial types follow this scheme. Since most of the work on fimbriae has concentrated on species in the Enterobacteriaceae, a brief overview of the major types found in this family will be discussed.

The surface fibrils of enterotoxigenic *E. coli* have been separated into three major classes based on morphology and function. The first class includes type 1, 987P (P fimbriae), CFA1, CS1, and CS2 fimbriae. These fimbriae are all rigid with a diameter of approximately 7 nm (Gaastra and deGraaf, 1982). The type 1 fimbrial structure is helical with 3.125 subunits per revolution and a subunit pitch distance of 2.3 nm (Levine et al., 1984). As well, the type 1 fimbriae appear to be hollow with a 2.3 nm axial hole present in the centre of the fibril. Type 1 fimbriae are widely distributed on strains of *E. coli.* It has been estimated to be present on as many as 70% of all wild type strains (Klemm, 1985). These fimbriae are able to bind to D-mannose residues on eukaryotic cells including erythrocytes (Clegg and Gerlach, 1987). When bacteria with type 1

fimbriae are mixed with erythrocytes, the cells clump together or agglutinate. This hemagglutination is also referred to as being mannose sensitive, since the addition of free mannose blocks the agglutination through competition for the fimbrial binding sites.

P (P blood group antigen), Pap (pyelonephritis-associated fimbriae), 987P and F41 (named according to the *E. coli* strain) fimbriae are found on uropathogenic strains of *E. coli* (Klemm, 1985). These fimbriae bind to the digalactoside a-D-Gal-(1-4)-β-D-Gal sequences of P blood group antigens present on human cells (Rhen et al., 1983).

The K88, K99, F41 and CS3 fimbriae form a second class. These fimbriae are characterized by their thin diameter (2-5 nm) and flexible morphology (Gaastra and deGraaf, 1982). These fimbriae may also be referred to as fibrillae (Levine, 1984). Both the first and second classes of fimbriae are peritrichously arranged in numbers ranging from 100-1000 per cell.

In contrast, the sex pili, the third class of fibril, are present in much smaller numbers. There are two major types of sex pili that have been distinguished: F and I-like pili. I-like pili are associated with the colicin factor and I-like R factors. In general, the I-like pili are shorter and are recognized and bound to by different bacteriophages than the F-like pili (Ottow, 1975). F pili are generally 1-2 μm long, but fibrils up to 20 μm have been observed (Brinton, 1965). Unlike fimbriae, the average number of sex pili present per cell ranges from 0.5 to 1.5 (Ippen-Ihler and Minkley, 1986). F pili are approximately 8 nm in diameter and have a 2 nm central axial hole (Brinton, 1965). X-ray fibre diffraction patterns indicate that F pili are related by a five fold rotation about the helix axis. The helix symmetry is 25 units in two turns of the helix. The pitch is 16

nm and the crystallographic repeat is 32 nm (Marvin and Folkhard, 1986). Terminal knobs may also be present. It is not clear whether these are naturally occurring structural entities of the pilus or fixation artifacts, since treatment with mild denaturants also produces terminal knobs. In either case, these structures probably represent disordered pilin subunits (Ippen-Ihler and Minkley, 1986).

There are an ever increasing number of fimbrial types being identified on various prokaryotic species. It is tempting to describe all of these in detail. However, most of the fimbrial systems to date have similar genetic organizations, morphology, assembly mechanisms and functions. Therefore, only the major fimbrial types will be discussed as general examples.

Biochemistry

Type 1 fimbrial proteins have been isolated from *E. coli* (Salit and Gotschlich, 1977), *Salmonella typhimurium* (Korhonen et al., 1980), *Klebsiella pneumoniae* (Fader et al., 1982), and *Serratia marcescens* (Kohno et al., 1984). The purified type 1 fimbriae were found to be composed entirely of protein, with no detectable levels of carbohydrates, lipids or nucleic acids present (Brinton, 1965). Fimbrial protein monomers are able to reassemble themselves into characteristic type 1 fimbriae confirming that these proteins are solely responsible for fibril formation (Eshdat et al., 1981). The molecular weights of the fimbrial proteins are 17000, 21000, 19500 and 19000 daltons for *E. coli* , *S. typhimurium* , *K. pneumoniae* and *S. marcescens* , respectively. The fimbrial proteins of Pap, CFA1, and K99 fimbriae were also found to be

similar in size. In contrast, the molecular weight of the K88 fimbrial subunits is 27500 daltons (Klemm, 1985). While there are general similarities in size, there is considerable variation in the amino acid compositions of these fimbrial proteins. This variation even extends to comparisons between fimbrial subunits of type 1 fimbriae amongst different enteric species (Finlay and Falkow, 1989). However, there are amino acid homologies at the amino and carboxyl termini of the type 1, Pap and K99 fimbriae (Mooi and deGraaf, 1985). Furthermore, the proteins are composed of approximately the same number of amino acids and cysteine residues are located at approximately the same positions. The cysteine residues are thought to form intra-chain disulfide bonds (Isaacson and Richter, 1981). In order to disrupt fimbriae, harsh treatments such as boiling in acid or exposure to saturated guanidine hydrochloride are required (Eshdat et al., 1981; Dodd and Eisenstein, 1982). It is thought that the conserved regions of the fimbrial subunits are necessary for the subunit-subunit binding. This is especially true of the C-terminus which is generally rich in hydrophobic amino acids. Thus, these are likely to be present within the fimbrial structure. In *Pseudomonas* fimbriae and the type 1 fimbriae of *E. coli* , tyrosine residues are probably present at the subunit-subunit interface . It is thought that this aromatic amino acid is responsible for the subunit-subunit binding (Watts et al.,1983; McMichael and Ou, 1979).

Although isolated fimbrial subunits can reassemble to form characteristic type 1 fimbriae, there are minor proteins present as well. This was first determined by the separation of the gene products necessary for fimbrial assembly from those required for adhesion (Minion et al., 1986; Norgren et al., 1984). Norgren et al. (1984) had isolated a

chromosomal DNA fragment that contained all the necessary information for normal Pap fimbriae formation. A series of mutant bacteria with Tn5 insertions in this fragment were produced. Several bacteria exhibited normal fimbrial production, but lacked the ability to agglutinate erythrocytes. Similar results have also been obtained for type 1 (Minion et al., 1986) and S fimbriae (Moch et al., 1987). Thus, the erythrocyte agglutinating principle was found to be a separate class of proteins termed "adhesins". Unlike the fimbrial structural subunits which exhibit considerable variation, the adhesins of the various fimbrial types are conserved amongst members of the Enterobacteriaceae (Abraham et al., 1988; Finlay and Falkow, 1989). Recently, Stromberg et al. (1990) examined two Pap-G and two Prs-G adhesins of P fimbriae. These adhesins are differentiated by their abilities to bind to separate Galα 1-4 Gal isoreceptors. In some *E. coli* strains, there may be two separate pap operons present. These may be expressed at the same time, thus producing strains containing fimbriae with binding affinities for at least two different receptors. Furthermore, these researchers found that even a single amino acid substitution was capable of altering the adhesin binding specificity. They speculated that these amino acids must be integral to the receptor binding domain. Thus, simple amino acid substitutions may result in the ability of the bacterium to adhere to novel sites.

Even though adhesins have been isolated and characterized, the adhesive role of the structural fimbrial subunits cannot be ruled out entirely. For example, the K99 structural subunit also functions as an adhesin (Jacobs et al., 1987). Poor characterization of this function in other fimbrial types is partly due to wide variations in the amino acid sequences of the structural fimbrial proteins (Finlay and Falkow, 1989).

F pili are composed solely of a 7200 dalton protein (F-pilin). F-pilin is 70 amino acids long and is acetylated at the N-terminus (Frost et al., 1984). Additionally, F-pilin appears to be glycosylated and phosphorylated (Armstrong et al., 1981). The structural gene traA has been sequenced and indicates that a 51 amino acid leader sequence is present on the immature pilin protein (Frost et al., 1984). This leader sequence has a typical hydrophobic core and signal peptidase recognition site for cleavage at the F-pilin N-terminus.

Assembly

One of the more interesting aspects of fimbriae is the mechanism of assembly. The best characterized fibrillar organelle to date is the flagellum, which like fimbriae is composed of monomeric subunits. These subunits are polymerized at the tips of the flagellum. Monomers are most likely transported through the flagellum's large hollow core (Lowe et al., 1987). Unlike flagella, fimbriae have a much smaller axial hole (2 to 2.5 nm). Therefore, this channel is not thought to be the conduit for their assembly (Brinton, 1965). Lowe et al. (1987) have shown that type 1 fimbrial assembly occurs at the base and not at the tips. This was determined by pulse-labelling *E. coli* with newly regenerating fimbriae with anti-fimbrial antibodies conjugated to gold particles. After regeneration, the gold labelling was present at or near the ends of the fimbriae. Thus, new fimbrial subunits are added at the base of the fimbriae.

Antibodies raised against the various P-fimbrial proteins (PapA-H) bind two minor subunits PapE , PapF and the adhesin PapG at the fimbrial

tip (Lindberg et al., 1987). This location of the adhesin at the fimbrial tip is reinforced by previous findings that agglutination of erythrocytes by isolated P-fimbriae is enhanced by the addition of Mg^{2+} (Lindberg et al., 1984). Mg^{2+} aggregates detached fimbriae. This process produces multivalent particles, which may form cross bridges between the erythrocytes, resulting in agglutination. If fimbriae had multiple adhesion sites along their length, then the addition of Mg^{2+} would not be necessary for agglutination of erythrocytes by detached fimbriae (Lindberg et al., 1987). Obviously, when the fimbriae are attached to the bacterium a multivalent condition exists.

The assembly of the K88 and P-fimbriae are the best characterized systems to date. The assembly of P-fimbriae is described below. The assembly of the fimbria occurs where the outer membrane protein, Pap C, is located. This protein forms the base of the fimbria (Baga et al., 1987). The various fimbrial subunits PapA, PapG, PapF, PapE and PapH are transported to this site by the periplasmic PapD protein. These proteins each form a separate transient complex with PapD. $PapE^-$ mutants form normal fibers, but the association of the PapG (the adhesin) with the fimbriae is weakened (Lindberg et al., 1984). PapF appears to be involved in initiating and limiting fimbria formation, since fimbriation is drastically reduced without it (Lindberg et al., 1984). The PapG, PapF and PapE proteins form the tip of the fimbria. Once their assembly is complete, the major structural subunit PapA is assembled into the fimbria. The number of PapA subunits added appears to be regulated by PapH. $PapH^-$ mutants have longer fimbriae than do the wild type strains (Baga et al., 1987). The PapH protein is very similar to PapA, except for the presence of a 14 residue proline rich sequence extending from the NH_2^- terminus (Baga et

al., 1987). Proline rich regions in staphylococcal protein A (Guss et al., 1984) and streptococcal M protein (Hollingshead et al., 1986) have been implicated to bind to the cell wall. Since fimbriae detach more readily in PapH$^-$ strains, it appears that the PapH protein also functions to secure fimbriae to the cell wall (Baga et al., 1987).

The assembly of pili is not as well characterized as the assembly of P or K88 fimbriae. The individual functions of the tra gene products in pilus assembly have not been determined. However, it appears that unlike the static fimbrial structures, F pili may have the ability to depolymerize, thereby enabling pili to retract. This ability was proposed based on the following evidence: 1) during phage infection F pili disappear and 2) rapid wall-to-wall contact occurs between conjugating bacteria (Ippen-Ihler and Minkley, 1986; Jacobson, 1972). It is unknown whether F pilus assembly occurs at the base or the tip.

Genetic Organization

The genes involved in the synthesis of type 1, P and F41 fimbriae all reside on the chromosome. However, the K88, K99, CFA/1, CS2 and CS3 fimbriae are all encoded for by genes harboured on plasmids (Mooi and deGraaf, 1985).

The approximate location of the type 1 fimbriae locus was first discovered by Brinton et al. (1961). A more precise estimation of location was offered by Maccacaro and Hayes (1961) with interrupted mating experiments between Fim$^-$ and Fim$^+$ strains of *E. coli* . They mapped the locus between the thiamine and threonine loci. The map below indicates the position of the fimbrial locus in relation to neighbouring loci. The

distances between loci are shown in a time scale in minutes (from Ottow, 1975).

O - met - thi - fim - thr - leu - az

2 6-7 2-3 1 0.5

Freitag and Eisenstein (1983) have pinpointed the location of the fim locus to 97.6 minutes on the chromosome.

The organization of the pap gene cluster has been well characterized (Lindberg et al., 1987). In other fimbrial gene clusters, there are approximately the same number of genes which may even be arranged in a similar genetic order (Riegman et al., 1990). The pap gene cluster is shown below (from Lindberg et al., 1987).

The pap gene cluster is composed of nine genes that are involved in fimbrial biosynthesis. Even though in other fimbrial gene clusters the organization of genes may be similar, the gene products from one fimbrial gene cluster may not be functional in another (Riegman et al., 1990). Furthermore, sequence homology of the various gene clusters is very low (Ott et al., 1988). Due to this low level of DNA sequence homology, it is uncertain whether these clusters have undergone divergence. Instead, they may be the result of convergent evolution (Gerlach et al., 1989).

An interesting mechanism for the control of fimbrial production has been observed. Cells were found to oscillate between states of fimbriation

and non-fimbriation at a frequency of about one switch per 1000 cells per generation (Eisenstein, 1981). Thus, fimbriae expression undergoes phase variation. This "on" and "off" mechanism of expression occurs at a much higher rate than the spontaneous mutation rate. Furthermore, the expression is reversible (Eisenstein, 1988). In *E. coli* , phase variation is the result of the inversion of a 314-bp region of the fimA (major subunit) promoter (Abraham et al., 1985). This inversion is mediated by fimB and fimE which are located directly upstream of fimA. The fimB gene product mediates the inversion of the invertible segment to the "on" position, while fimE is responsible for inversion of the segment to the "off" position (Klemm, 1986). The DNA sequences of fimB and fimE showed sequence homology with the putative active sites of the integrase class of site-specific recombinases (Dorman et al., 1987). Integrases are involved in reciprocal recombination between specific sites (att) on both phage (eg. λ) and bacterial chromosomes (Nash, 1975). As with the λ Int protein (an integrase), fimA phase variation is dependent upon the presence of the integration host factor (IHF). At either end of the invertible DNA segment are sequences that are homologous to the recognition sites for IHF. Thus, it appears that IHF mediates the intramolecular recombination event between these sites (Dorman et al., 1987). The basis for this control of gene expression may be to increase the virulence of the organism depending on the environment. In *Proteus mirabilis* , the fimbriated strains are more virulent in the colonization of the host, since the fimbriae are required for attachment to host tissues. However, once the bacteria have invaded, then the non-fimbriated strains become more virulent. Since fimbriae are involved in adherence, then they will probably bind to leucocytes as well. This would eliminate the fimbriated bacteria from the

invasive population. This would leave the non-fimbriated strains (Silverblatt and Ofek, 1979 from Eisenstein, 1988; Eisenstein, 1982).

In *Moraxella bovis* , the inversion of a 2 kb segment of DNA results in the alternate expression of two serologically distinct fimbrial subunit genes. Within this DNA segment is all of the information required for expression of two fimbrial proteins α and β , with the exception of the promoter and part of the amino terminal. In one orientation, the α fimbrial gene lies next to the promoter and is expressed. The β-gene cannot be expressed since it has no promoter. When the DNA sequence is inverted the reverse is true (Marrs et al., 1988).

The *M. bovis* fimbrial proteins share extensive N-terminal amino acid sequence homology with fimbrial proteins from *Moraxella nonliquefaciens, Neisseria gonorrhoeae , N. meningitidis , Bacteroides nodosus , Pseudomonas aeruginosa* and *Vibrio cholerae* (Elleman, 1988). The first 32 amino acids are nearly identical and all of the species contain N-methyl phenylalanine as the first residue. Like *M. bovis* , *N. gonorrhoeae* can also vary the antigenicity of its fimbrial subunits. However, it performs this task in a very interesting manner, and to a much greater extent. A single cell of one gonococcal strain is capable of giving rise to progeny that express fimbrial proteins which are antigenically unique. The fimbrial genes of these bacteria can be divided into constant (C), semivariable (SV) and hypervariable (HV) regions (Hagblom et al., 1985). The C region is conserved and encodes the first 50 amino acids of the amino terminus. In the SV region (amino acids 54 to 114), single base pair changes are observed, which result in single amino acid substitutions. Most of the variation occurs in the HV region (amino acid 115 to the carboxy-terminus). In this region, insertions and deletions of 1-4 amino

acids, as well as single base pair changes, are observed. This region is flanked by the conserved sequences containing the cysteine residues responsible for the disulphide bridge. Between these residues resides the most antigenic portion of the fimbrial protein (Nicolson et al., 1987). Thus, the presence of an HV segment of amino acids in this region would lead to the observed antigenic variation. This variation is hypothesized to occur by recombination with other silent fimbrial sequences present in the genome. To date, seven silent fimbrial loci have been identified. Moreover, recombination could occur between separate silent loci giving rise to a new silent fimbrial gene (Seifert and So, 1988). It is thought that the conserved regions of the fimbrial gene in the expression site serve as crossover sites for recombination (Seifert and So, 1988). Furthermore, this recombinational event is non-reciprocal. This event is an example of gene conversion. Southern hybridization studies did not identify the presence of the original sequence after recombination (Seifert and So, 1988).

Recombination may occur between the active fimbrial locus and DNA sequences from other gonococci. In actively growing cultures, it is natural to find gonococci that have autolysed (Hebeler and Young, 1975). Moreover, it has been shown that fimbriated gonococci have transformation frequencies for auxotrophic markers of about 10^{-2} to 10^{-3}. The transformation frequency is expressed as the percentage of colony-forming units transformed. Non-fimbriated cells have transformation frequencies of about 1000 to 10000 times lower (Sparling, 1966). It has also been shown that mixed gonococcal cultures may exchange chromosomal markers (Sarubbi and Sparling, 1974 from Seifert and So, 1988). Thus, the gonococci can uptake DNA from other lysed strains and incorporate them into their genome. In fact, Seifert and So (1988) have

reported that sequences obtained by DNA uptake give up to 100 times the level of recombination than that which occurs from the silent loci present in the cell. Recombinational events between the silent loci present and the DNA sequences taken up are also possible (Seifert and So, 1988). Thus, variation in fimbrial genes could occur by replacement of an active gene by transforming DNA, or replacement of silent genes followed by second recombinational event between an active gene and "new" silent genes.

Infectious, transmissible elements were found in "fertile" strains of bacteria in the early 1950's (Cavalli-Sforza et al., 1953; Hayes, 1953). These elements are plasmids. At least 60 genes are present on the F plasmid which is a circular, covalently closed dsDNA molecule of about 100 kb pairs in length (Johnson and Willetts, 1983). Most of the genes known to be involved in DNA transfer (tra genes) are clustered in a 33.3 kb region of the plasmid. Presently 22 cistrons that are required for the transfer of F plasmids to new hosts have been identified. Additional cistrons present on both the F plasmid and bacterial chromosome are likely necessary for F transfer as well (Ippen-Ihler and Minkley, 1986).

The gene products of the tra genes may be involved in four different functions associated with F transfer: 1) the synthesis of F pilin, 2) the production of "surface exclusion" proteins which prevent self-mating, 3) the expression of genes that stabilize the donor-recipient surface associations, and 4) the expression of genes associated with the functions that are involved in DNA transport from the donor to the recipient strain (Ippen-Ihler and Minkley, 1986).

Function

I. Adhesion

Adhesion of bacteria via fimbriae to erythrocytes has been known for over thirty years (Duguid et al., 1955). This characteristic has been used in their classification as discussed earlier. Fimbriae also mediate bacterial adherence to mucosal surfaces. They are thought to bind to the carbohydrate component of glycoconjugates present on the cell surfaces of their host (Klemm, 1985). Recently, site-directed mutagenesis of the type 1 fimbrial gene of *E. coli* K1 has been performed. Results showed that fimbriae were required for colonization of the alimentary tract, but were not necessary for invasion of the bloodstream (Bloch and Orndorff, 1990). Furthermore, the adhesion of *Pseudamonas aeruginosa* to respiratory epithelial cells has been blocked with monoclonal antibodies directed against fimbriae (Doig et al., 1990). Adherence of a pathogen to host tissue mediates the following: 1) the maintenance of a close approximation to the epithelium; this might allow for more effective delivery of toxins to the host and may enhance the uptake of nutrients from the host (Middledorp and Wilholt, 1981; Zafriri et al., 1987) and 2) the ability of bacteria to avoid the cleaning mechanisms and frequent flushings associated with infection of higher animals (Klemm, 1985). The consequences of these facets are that fimbriated bacterial strains grow more significantly and are approximately 100 times more virulent than non-fimbriated strains (Eisenstein, 1988).

II. Transformation

N. gonorrhoeae is naturally competent for DNA transformation, especially for its own DNA (Seifert and So, 1988). Remarkably, fimbriated gonococci exhibit transformation frequencies of 1000 to 10000 times that of non-fimbriated gonococci (Sparling, 1966; Seifert et al., 1990). This uptake of DNA was shown to aid in the generation of antigenically variant fimbrial proteins, which help the gonococci in avoiding the host immune response (Seifert and So, 1988).

III. Motility

Along with *N. gonorrhoeae* , other bacterial species with polar mePhe fimbriae also exhibit "twitching motility". When grown on agar, spreading zones are observed. It appears that by twitching, the cells are able to move across the surface of the agar (Henrichsen, 1983). However, Henrichsen (1975) has also pointed out that this phenomenon could be explained by Brownian movement or electrostatic repulsive forces.

IV. Conjugation

It is generally accepted that F pili are necessary for the initial contact between DNA donor and recipient bacteria (Achtman et al., 1971; Brinton et al., 1964). Conjugation does not take place if F pilus synthesis is blocked by mutation (Willetts and Skurray, 1980), growth conditions (Walmsley, 1976), chemical treatment (Achtman et al., 1978) or if pili are mechanically removed (Novotny et al., 1969). Furthermore, elements such as F-specific DNA phages and Zn^{2+} ions which bind to the pilus tip, also block conjugation (Ou, 1970; Brinton, 1965). However, it is unclear

whether or not pili play a larger role in DNA transfer. Brinton (1965) hypothesized that pili are directly involved in DNA transfer. Once pilus contact occurs, a signal is induced which initiates the transfer of the F plasmid. DNA transfer was hypothesized to occur through the hollow core of the pilus. This was reinforced experimentally by Ou and Anderson (1970) who isolated recombinants from strains that remained at least 1 mm apart. However, no evidence for the association of F pili and DNA has been observed. Curtiss (1969) and Marvin and Hohn (1969) have suggested that the retractile capabilities of pili bring the donor and recipient cells close together. Once cell wall-to-wall contact occurs, a cytoplasmic bridge is produced which facilitates DNA transfer. Ippen-Ihler and Minkley (1986) have pointed out that pili could still be an integral part of the cytoplasmic bridge through which DNA is transferred. Thus, an extended pilus may be able to serve as the cytoplasmic bridge in an unstable manner.

Fungal Fimbriae

It is important to note that most of the work pertaining to fungal fimbriae, has been performed using *Ustilago violacea*. This fungus is a parasite of the carnation family (Caryophyllaceae), with physiological races specific for various host species (Zillig, 1921; Liro, 1924 and Goldschmidt, 1928). Most of the work performed on fimbriae was accomplished using an isolate obtained from *Silene alba* (White Campion) (Day and Cummins, 1981).

Structure

Fimbriae of *U. violacea* are long fibrils with lengths of 0.5 to 20 mm and a diameter of 7 nm (Poon and Day, 1974,1975; Gardiner and Day, 1985). The fibrils do not branch or taper and appear to be curved and flexible. They are thought to originate below the cell wall and penetrate it (Poon and Day, 1974,1975). This is shown in freeze etch preparations by the presence of particles 7 to 10 nm on the outer surface of the plasma membrane, mirrored by similar projections on the inner surface of the cell wall. Further evidence comes from sections through the cell wall, which show the presence of 7-10 nm fibrils traversing the whole cell wall (Poon and Day, 1974,1975). Thus, it appears that fimbriae are firmly anchored in the cell surface.

Diffraction analysis of electron micrographs has shown that each fiber is made up of a helical coil (Gardiner et al., 1979). Staining with phosphotungstic acid did not show the presence of a hollow structure as in the fimbriae of *Ecsherichia coli* (Poon and Day, 1975; Brinton, 1965).

Thus, the fimbriae of fungi and bacteria share similar morphological features, but differ in their structural make-up.

The distribution of fimbriae in *U. violacea* is thought to be uniform over the entire cell wall, with 0 to 200 fibrils per cell (Poon and Day, 1974). The number of fibrils present is dependent upon culture conditions, since fungi grown in broth cultures are more fimbriated than corresponding colonies grown on nutrient agar (Poon and Day, 1974). In addition, logarithmic phase cultures are more fimbriated than stationary phase cultures which lack fimbriae (Poon and Day, 1974). However, variations in the amount of fimbriae present have not been found to be correlated with any particular stage of either the cell or life cycles, except for their absence on the thick-walled teliospore (Poon and Day, 1975; Gardiner and Day, 1985). It is also interesting to note that 1 to 4 knobbed fimbriae may also be present per cell (Day and Poon, 1975). This is particularly interesting, since similarly knobbed pili on bacteria are involved in sexual conjugation.

Isolated fimbriae are very stable and will maintain their shape and antigenicity after exposure to temperatures up to 100° C (Gardiner and Day, 1985). However, the fimbriae present on the fungus are maintained within a smaller temperature range (10° to 25° C) than the cells growth range (1° to 28° C). Since fimbrial subunits are very stable, this observation is postulated to be a result of regulation of synthesis, rather than due to a heat sensitive component of fimbriae (Gardiner and Day, 1985). In addition, cells treated over a pH range of 3 to 9 retained their fimbriae, reinforcing the stable nature of their structure (Gardiner and Day, 1985).

Fimbriae can be removed by mechanical agitation, heat, sonication or centrifugation through a viscous liquid (Poon and Day, 1974). They reappear about one hour after removal and reach normal lengths in less than 5 h. The apparent growth rate is 1 to 2 μm/h (Poon and Day, 1974,1975). It appears that this growth phenomenon occurs under normal cell growth conditions. In most culture media detached fimbriae have been observed, indicating that they can be shed and replaced (Poon and Day, 1974). Other regeneration experiments have focused on the inhibition of fimbrial growth with the blocking agents cycloheximide (an inhibitor of eukaryotic translation), chloramphenicol (an inhibitor of prokaryotic and mitochondrial translation) and rifampin (an inhibitor of eukaryotic and prokaryotic transcription). Regeneration only occurred in cultures with chloramphenicol, indicating that regeneration may be dependent upon both translation and transcription of nuclear genes. Unlike bacteria, there appears to be no free pool of subunits (Poon and Day, 1975).

Fimbriae are proteinaceous, since they are digested by proteases but not by other enzymes (Day and Poon, 1975). SDS-polyacrylamide gel electrophoresis of the dissociated proteins from isolated fimbriae indicate that the proteins are of one molecular weight, 74 000 daltons (Gardiner and Day, 1985). When these proteins are separated on an isoelectric focusing gel, two major bands are observed, one at pH 6.8 and the other at pH 7.0 (Gardiner et al., 1979). Analysis of these proteins suggested that one was phosphorylated (Day and Cummins, 1981). Further analysis has indicated that fimbriae may be composed of three to eight 74 kd proteins. The cause of the variation in the number of proteins observed between preparations is unknown. However, this variation is not dependent upon mating type, temperature or growth phase of the cells

(Castle et al., manuscript in preparation). It has been suggested that these proteins may result from a single nascent polypeptide and that the differences in their isoelectric points may be the result of posttranslational modifications. For example, the isoelectric point of a protein would be altered with the addition of phosphate or a methyl group. Although previous findings have indicated that the fimbrial proteins are entirely proteinaceous (Poon and Day, 1974; Gardiner and Day, 1985), recent work has shown that fimbrial proteins are glycosylated (Castle et al., manuscript in preparation).

The 74 kd proteins are probably the major structural components of the fimbriae of *U. violacea* . The isolated proteins from both SDS and IEF gels can be reassembled *in vitro* to resemble typical fimbriae. Moreover, these intact fimbriae will attach to cells from which the fimbriae have been removed (Gardiner and Day, 1985). However, this does not eliminate the possibility that other macromolecules may be integral to the structure and function of the fibril. In bacteria, fimbriae usually have separate structural and adhesin components (Minion et al., 1986; Norgren et al., 1984).

Serology

Antibodies produced against the 74 kd proteins isolated from the fimbriae of *U. violacea* (AU) and also from the basidiomycetous yeast *Rhodotorula rubra* (AR) have been used in serological studies with these and other fungi (Gardiner et al., 1981,1982). These studies, coupled with electron microscopy, showed that a number of species in the Ustilaginales and related heterobasidiomycetous yeasts have fimbriae. Cells of all

members of the Ustilaginales possessing fimbriae agglutinated with AU. However, no agglutination was observed with heterobasidiomycetous yeasts outside of this order. Thus, it appears the Ustilaginales share a similar epitope or protein subunit on their surfaces, while other heterobasidiomycetous yeasts have a distinct subunit type (Gardiner et al.,1981) . Basidiomycetous (long fibrils 10-20 μm) and ascomycetous yeasts (short fibrils 0.5-1 μm) tested for agglutination with both the AU or AR antibodies yielded all possible outcomes: 1) no agglutination with either antiserum; 2) agglutination with AU, but not with AR; 3) agglutination with AR, but not with AU; and 4) agglutination with both antisera (Gardiner et al., 1982) . Furthermore, the AU antiserum agglutinated cells of the algal species *Chrysochromulina breviturrita* and some other species in the Prymnesiophyceae and Chrysophyceae. However, the antibody did not agglutinate cells of any other alga. It has been suggested that these observations may indicate a previously unrecognized relationship between the members of these algal groups and fungi (Day et al., 1986b) .

The authors of these papers acknowledge that there may be problems in the interpretation of the agglutination tests. Fungi that may have had short and sparsely distributed fimbriae may not have reacted as strongly or at all with the antibodies. Likewise, these fimbriae may have been limited to a specific stage of the fungus' life cycle or to a specific environmental condition (Day et al., 1986b) . This problem is exemplified by *Ascocalyx abientia* which is not agglutinated by AU nor AR (Gardiner et al., 1981, 1982). However, protein A-gold labelling studies showed that both AU and AR recognized surface antigens in thin sections of this yeast (Benhamou et al., 1986). It is important to note that *R. rubra* is not

agglutinated by AU nor is *U. violacea* agglutinated by AR. Since these antibodies are polyclonal the epitopes they each recognize must be distinct. If they do recognize some of the same epitopes then these antibodies must be a minor component of the polyclonal mixtures. Using protein-A gold labelling techniques, Benhamou et al. (1986) reported that AU and AR both recognized antigens in *Ascocalyx abietina*, but in different cellular locations. They have hypothesized that there might be at least two distinct fimbrial protein species, one recognized by AU and the other by AR. In addition, there may be other protein species that AU and AR do not recognize. In order to test this hypothesis, monoclonal antibodies recognizing a single epitope must be obtained and tested against the fimbrial antigens of this species (Benhamou et al., 1986).

Several filamentous fungi from all divisions were observed to be fimbriated with electron microscopy (Day and Gardiner, 1988). Furthermore, immunofluorescent staining with AU, indicated that all of these strains shared surface antigens with *U. violacea* . Thus, all of these fungi probably produce a conserved fimbrial subunit. It is unusual that many fungi from all divisions would share a fimbrial antigen as determined in immunofluorescent studies, yet most of the basidiomycetous and ascomycetous yeasts did not agglutinate with AU. This apparent discrepancy may be due to different sensitivities of the two procedures.

Function

When one reviews the structural details of the fimbriae of *U. violacea* , it is apparent that they share similar morphological traits with bacterial

fimbriae and pili. This observation incites speculation that fungal fimbriae might be functionally equivalent to bacterial fimbriae and pili. In bacteria, fimbriae and pili are known to be involved in adhesion, DNA uptake, twitching motility and conjugation (Duguid et al., 1955; Brinton et al., 1964; Sparling, 1966 and Henrichsen, 1975).

In fungi, the role of fimbriae is not as well understood. In *Saccharomyces cerevisiae*, flocculation is dependent upon the presence of fimbriae, suggesting that they have an adhesive function (Day et al., 1975). Other evidence from *Saccharomyces carlsbergenesis* suggests that the fimbriae may also be involved with the secretion of proteins. This could be the secretion of the mannoprotein complex present on the cell walls of flocculant strains (Day et al., 1975). Thus, fimbriae may only be indirectly responsible for adhesion.

In *Candida albicans* , a mannoprotein polymer forms a "fibrillar layer" on the cell surface (McCourtie and Douglas, 1981). These fibrils have been shown to mediate the attachment of *C. albicans* to mucosal surfaces (Marrie and Costerton, 1981) and renal epithelium (Barnes et al., 1983). In addition, these fibrils enhance the virulence of the organism in mice (McCourtie and Douglas, 1984). The adhesion of *C. albicans* to buccal and epithelial cells can be inhibited with carbohydrates (Critchley and Douglas, 1987b). Critchley and Douglas (1987a) discovered that there were two major types of adhesins; one that adheres to L-fucose and another that adheres to N-acetylglucosamine. Thus, it appears that like bacteria, *C. albicans* adheres to a glycoconjugate on the cell surface of its host. Since, adhesion is dependent upon the presence of the mannoprotein fibrils, the adhesins may be a component of these fibrils. Houston and Douglas (1989) have indicated that the production of fibrils

may also assist this fungus in evading intracellular phagocytosis. It appears that secretion of the mannoprotein complex (fimbriae) interferes with phagocyte function (Wright et al., 1983).

Most of the work related to function has focused on the role of fimbriae in conjugation in *U. violacea*. No direct proof that fimbriae are required for conjugation has been put forth, but several experiments provide strong circumstantial evidence. In particular, experiments have shown that both fimbriation and conjugation share the following: the same maximum temperature tolerance, identical responses to shifts in temperatures and similar responses to enzyme treatment (Day and Poon, 1975). Studies on the effects of inhibitors of transcription and translation on mating cells led to the hypothesis that an exchange of information in the form of macromolecules takes place between the two mating types a1 and a2, before assembly of the conjugation tube (Poon and Day, 1974; Day and Poon, 1975). This mutual exchange may be required for development of the conjugation tube. It may occur at an early stage in conjugation, while the cell walls of both participants appear to be fully intact when examined microscopically (Day and Cummins, 1974). The only possible connections between the cells at this stage are fimbrial (Day and Cummins, 1974, 1975). Since a proportion (4 to 5%) of cells are observed to conjugate at distances of 4 to 20 μm without any direct cell wall to cell wall contact, fimbriae probably play a role in cell communication. Fimbriae may serve as conduits for transfer of information between appropriate mating partners. The receptive cells respond by initiating conjugation tube formation. As an alternative one might suggest that the path of conjugation tube formation may follow the diffusion gradient of a substance released by the mating cells. However,

cells do not always mate with the closest possible cell of opposite mating type. Occasionally, conjugation tubes will bypass one compatible cell for another further away (Day and Poon, 1975). It is difficult to explain this observation with a diffusion gradient model.

Finally, studies on the role of fimbriae during plant pathogenesis have been initiated recently. Using protein A-gold immunocytochemical labelling, fimbrial antigens were identified in the cytoplasm of host cells which were several micrometers from the fungal hyphae (Day et al., 1986a; Svircev et al., 1986). However, these observations could not reveal whether the fimbriae were intact within the cells, or present as dissociated proteins which may have penetrated the host cell accidentally during preparation (Day et al., 1986a; Svircev et al., 1986). There is also a possibility that the host cell may have produced an antigenically similar protein in response to the fungal infection (Svircev et al., 1986). Alternatively, a greater intensity of labelling has been observed on the fungal cell walls of *Ophiostoma ulmi* in infected elm tissue as compared to when the fungal cells were grown axenically (Day et al., 1986a; Benhamou et al., 1986). This apparent increase in fimbrial protein production suggests that fimbriae are required by the cells to perform some function while within the host tissue. These functions could include recognition, adhesion to the host, determination of the nutrient location within the host and possibly the excretion or absorption of substrates involved in infection of the host.

Unfortunately, the role of fungal fimbriae is based entirely upon correlations. There is no direct evidence for their role in any cellular process. This is partly due to the inability to produce stable fimbrial mutants. Furthermore, the fimbrial connections between cells and the

molecules that have been postulated to be transported by fimbriae have not been identified.

Materials and Methods

Culture Conditions

U. violacea 2716 (a2 mating type, pink colour and lys_2 (lysine-requiring)) and 1T10cb2 (a1 mating type, pink colour, arg_3 (arginine-requiring),cbr_1 (carboxin-resistant), his_1 (histidine-requiring) and lys_2 (lysine-requiring)) are auxotrophic strains obtained from UWO-1 (Gardiner et al., 1981). These strains were grown on Ustilago complete medium (UCM) at 22° C for 3-4 days and were maintained by subculturing approximately every two weeks. For liquid cultures, the strains were grown in Erlenmeyer flasks half filled with medium. UCM consisted of the following (Day and Jones, 1968):

Glucose	10 g
Peptone	10 g
Malt Extract	3 g
Yeast Extract	3 g
Agar (plate cultures)	20 g
Distilled Water	1 L

Schizophyllum commune 8-3 ($A_{43}B_{42}$ mating type and ade_5 (adenine-requiring)) was graciously given to us by Dr R. C. Ulrich (University of Vermont). This culture was maintained on Schizophyllum complete medium (SCM) through serial mycelial explants, approximately once a month. For liquid cultures, *S. commune* 8-3 was grown in a small volume of liquid medium. The mycelial thallus was harvested and macerated with an Omnimixer (setting 3) for a few seconds. This slurry was used as an inoculum for a larger volume of SCM. SCM consisted of the following

(Snider and Raper, 1958):

$MgSO_4 \cdot 7H_2O$	0.5 g
KH_2PO_4	0.46 g
K_2HPO_4	1.0 g
Peptone	2.0 g
Yeast Extract	2.0 g
Glucose	20.0 g
Agar	20.0 g
Distilled Water	1.0 L

Coprinus cinereus PR2301 (A_2B_3 mating type and met_1(methionine-requiring)) was graciously given to us by Dr B. C. Lu (University of Guelph). This culture was maintained on Coprinus complete medium (CCM) and was grown in liquid cultures in the same manner as was *S. commune* . CCM consisted of the following (Fries, 1953):

Glucose	20.0 g
Asparagine	2.0 g
Salt Solution (see below)	25.0 ml
Thiamin (10 % (w/v))	1.0 ml
Yeast Extract	0.75 g
Casein Hydrolysate (NBCo)	0.75 g
Malt Extract	0.60 g
Agar (plates only)	20.0 g
Distilled Water	1.0 L

Salt Solution:

Ammonium Tartrate	20.0 g/L
KH_2PO_4	40.0 g/L
Na_2HPO_4	90.0 g/L
$Na_2SO_4 \cdot 10H_2O$	11.2 g/L

Protein Isolation

Cellular protein

Mycelia of *S. commune* and *C. cinereus* were harvested from liquid cultures by filtration through cheese cloth, followed by repeated rinsings with distilled water. The samples were then freeze dried. Proteins were extracted by grinding the dried mycelia in a mortar and pestel with two parts acid-washed sea sand or silica gel, and one part cold TEPI (10 mM Tris, 1 mM ethylenediaminetetra-acetic acid (EDTA), 1 μM phenylmethylsulfonyl fluoride and 1 mM iodoacetamide) (pH 6.8). The resulting slurry was centrifuged at 10 000 X g for 10 minutes at 4° C and the supernatant plus any mycelial fragments were collected. The recovered samples were mixed vigorously with equal volumes of cold n-butanol to remove any lipids from the sample and to aid in the isolation any proteins associated with the cell membrane (Penefsky and Tzagoloff, 1971). After centrifugation at 1 000 X g for 10 minutes at 4° C, the bottom layer of buffer saturated with n-butanol was collected carefully, avoiding the central lipid layer. The samples were dialyzed against TE (10 mM Tris and 1 mM EDTA) (pH 7.5) at 4° C, freeze dried and suspended in a minimal volume of sterile distilled water. Samples were stored at -20° C.

U. violacea total protein was isolated from liquid cultures differently than *S. commune* and *C. cinereus* due to its tough cell wall. Cells were isolated by centrifuging liquid cultures of 2716 at 12 000 X g for 10 minutes at 4° C. The medium was poured off and the pellet of cells was suspended in distilled water. This washing of the cells was repeated two more times with the final pellet of cells being suspended in TEPI (pH 6.8) instead of distilled water. The cell suspension was passed through a French pressure cell at 12 000 PSI to break open the cells. The success of the pressure treatment was determined by examining the cells

microscopically. Cells were repeatedly passed through the pressure cell (usually two times), until the majority of the cells present were broken . The sample was centrifuged at 12 000 x g for 10 minutes at 4° C to remove any unbroken cells and cell debris. The supernatant was recovered and mixed with an equal volume of cold n-butanol. Subsequent steps in the protocol are identical to those outlined in the previous section, after the addition of n-butanol.

Fimbrial Protein

Cells of *U. violacea* 2716 were isolated and washed as outlined previously. Fimbriae were stripped from isolated cells in cold TEPI (pH 6.8) with a Sorval Omni Mixer at high speed (setting 10 for 1 minute). Samples were centrifuged at 12 000 X g for 10 minutes at 4° C to separate the intact cells from the stripped fibrils. The supernatant was recovered and freeze dried to concentrate the protein. After lyophilization, the samples were suspended in a minimal volume of sterile distilled water and stored at -20° C. Purity of the samples was determined by SDS-PAGE separation of the protein components.

Gel Electrophoresis

SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of the protein isolations was performed according to the procedure of Laemlli (1970), using a discontinuous polyacrylamide gel system (4%

stacking and 11% separating gels). The separating gel solution (0.375 M Tris, 11.1% acrylamide, 0.99% N, N'- methylenebisacrylamide (BIS), 0.1% SDS, 0.05% N, N, N', N' - tetramethylenediamine (TEMED), and 0.05% ammonium persulphate (APS) (pH 8.8)) was poured to a thickness of 0.75 mm. The freshly poured gel solution was carefully overlaid with a solution of n-butanol saturated with distilled water. Following polymerization (usually 45 minutes), the overlay was removed and the gel surface was washed thoroughly with distilled water to remove any residual n-butanol. The stacking gel consisted of 0.125 M Tris, 3.9% acrylamide, 0.347% BIS, 0.1% SDS, 0.1% TEMED and 0.05% APS (pH 6.8). The wells were produced with 10 well 0.75 mm thick combs. Following polymerization, the combs were removed and the wells were rinsed thoroughly with distilled water, then blotted dry with Whatman paper.

Protein samples were brought to 0.05 M Tris, 8% glycerol, 1.6% SDS, 4% 2-mercaptoethanol and 0.001% bromophenol blue (pH 6.8). These samples were heated for 5 minutes at 95° C and then loaded into the appropriate wells. The electrophoresis buffer was 0.007 M Tris, 1.44% glycine and 0.1% SDS (pH 8.3). Electrophoresis was carried out at 200 V (constant voltage) until the bromophenol blue reached the bottom of the gel (approximately 45 minutes). If the gels were stained with Coomassie blue, they were stained for one hour in a solution of 0.1% Coomassie blue R-250, 40% methanol and 10% acetic acid. Excess stain was removed by destaining the gels in a solution of 40% methanol and 10% acetic acid. If the gels were silver stained, the protocol of Merril et al. (1981) was followed.

Two-Dimensional Gel Electrophoresis

Two-dimensional (2D) gel electrophoresis was performed according to the O' Farrell (1975) procedure. Fifteen centimeter glass tubes (0.1 cm inner and 0.7 cm outer diameter) were sealed with dialysis tubing held onto the tubes with elastic bands and parafilm. The gel solution (48.6% urea, 3.54% acrylamide, 0.315% BIS, 2.03% Triton X-100, 2% ampholytes (pH 3/10), 0.1% TEMED and 0.013% APS) was carefully poured into the tubes to a height of 6.5 cm with a long needle. After the gels had polymerized (about one hour), the tubes were mounted into a tube gel apparatus and freshly degassed solutions of 0.1 N NaOH (catholyte) and 0.06% H_3PO_4 (anolyte) were placed in the top and bottom chambers respectively. The gels were prerun at 400 V (constant voltage) until the current decreased to 0 mA (about 15 minutes). Protein samples were suspended in 40% urea, 40% glycerol (v/v), 0.06% SDS, 0.53% ampholytes (pH 3/10) (v/v), and 1.33% Triton X-100 (v/v). These protein samples were applied to the top of the prefocused gels and were separated at 400 V (constant voltage) overnight, plus another 2 hours the next morning at 800 V (constant voltage) to sharpen the separation. Following isoelectric focusing (IEF), the tubes were removed from the tube gel apparatus. The acidic end of the gels was injected with a 0.05% bromophenol blue solution (w/v) using a Hamilton syringe and then placed on ice. The gels were extruded from the tubes with a 20 mL syringe. These extruded gels were equilibrated with reducing buffer (0.0625 M Tris, 5% 2-mercaptoethanol and 3% SDS (pH 6.8)) for 10-20 minutes. The gels were either stored frozen at -20° C, or directly laid onto the top of a SDS-polyacrylamide gel (4% stacking and 11% separating) as outlined in the SDS-PAGE protocol. The SDS-polyacrylamide gels were cast in the same manner as the one-

dimensional gels with the following exceptions: the gels were cast to a thickness of 1.5 mm to accomodate the IEF gel, and no wells were made in the stacking gel. Instead, the stacking gel was carefully overlaid with distilled water to produce a smooth surface allowing for good contact with the IEF gel. The gels were electrophoresed at 200 V (constant voltage) until the dye front reached the bottom of the gel (approximately 45 minutes). The gels were either stained with Coomassie blue, or transferred to nitrocellulose as outlined in the Immunoblotting procedure. Isoelectric points were determined by comparison with IEF standards (Bio Rad) which had been focussed in a separate gel. After IEF of the standards, the gels were stained in a solution of 0.1% Coomassie blue, 0.5% $CuSO_4$, 10% acetic acid and 27% isopropanol for one hour, and then destained in 0.5% $CuSO_4$, 10% acetic acid and 27% isopropanol until the protein bands could be visualized.

Immunoblotting

Following electrophoresis, gels (either 1-D or 2-D) were equilibrated for 10-30 minutes in Towbin transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol (v/v)) at 4° C (Towbin et al., 1979). Proteins were transferred from the gels to a nitrocellulose membrane with a Bio-Rad Transblot Cell. Transfer was performed at room temperature at 30 V (constant voltage) overnight, followed by another 2 hours at 60 V (constant voltage) the next day. Following transfer, the nitrocellulose membranes (blots) were washed for 10 minutes in Tris Buffered Saline (TBS: 20 mM Tris and 500 mM NaCl (pH 7.5) and then incubated in blocking solution (3% gelatin in TBS) for one hour. The membranes were

washed three times in TBS (15 minutes each). Next, the membranes were washed in TTBS (TBS with 0.05% Tween-20) for 15 minutes and then incubated with gentle agitation overnight in 1° antibody solution (1/1500 AU or NS, 0.05% sodium azide,1% gelatin in TTBS). The membranes were washed four times in TTBS (30 minutes each), once in TBS (30 minutes) and another two times in TTBS (30 minutes each). The membranes were incubated in 2° antibody solution (1/3000 goat anti-rabbit IgG alkaline phosphatase conjugate (Bio-Rad), 1% gelatin in TTBS) for 1 hour. Unbound and weakly bound 2° antibodies were removed in three washes of TTBS (15 minutes each). The membranes were washed in TBS twice (15 minutes each) to remove any residual Tween-20. Bound antibodies were visualized by incubating the blots in 0.1 M Tris, 1 mM $MgCl_2$, 0.03% nitro blue tetrazolium (NBT) and 0.015% Bichloro-indolyl-phosphate (BCIP) (pH 9.5) until colour development was complete. Excess colour development solution was removed in two washes with distilled water (10 minutes each). Immunoblots were blotted dry and stored on Whatman paper in the dark. The molecular weights of the proteins identified using this procedure were estimated using prestained low molecular weight standards (Bio-Rad).

Electroelution

Protein isolations of *C. cinereus* PR2301 were separated on an 11% SDS-polyacrylamide gel as outlined previously. The proteins were then isolated from the gels according to the procedure of Hanaoka et al. (1979) with minor modifications. Tubes with an inner diameter of 6 mm were constricted at the bottom end. The bottom end of the tube was sealed with

parafilm and a 50% solution of sucrose was pipetted to a height of 1 cm. The gel solution (6 M urea, 6% acrylamide, 0.16% BIS, 0.03%APS, 0.1% TEMED, 1 mM dithiothreitol and 40 mM glycine (pH 10.4)) was carefully poured on top of the sucrose solution. The gel solution was overlaid with distilled water and allowed to polymerize for 2 h. Following polymerization, the sucrose solution was removed and replaced with electrophoresis buffer (40 mM glycine and 1 mM dithiothreitol (pH 10.4)). A dialysis membrane was attached to the bottom of the tubes with elastic bands and parafilm. These gels were prerun for 2 to 3 hours at 2 mA/tube (constant current). The region of the SDS-PAGE gel in the 37 kD range was determined with prestained low MW standards. This section of the gel was isolated and chopped up into small fragments with a sterile razor blade. The gel fragments were mixed with 10% glycerol in electrophoresis buffer. This slurry was loaded into the tubes carefully. The elution was performed at 2 mA/tube (constant current) for 20 hours. After elution, proteins were dialyzed against TE (pH 7.5), concentrated by freeze drying and suspended in a small volume of a 5 mM phosphate buffer (pH 7.0). Success of the electroelution procedure was determined by the cytochrome C control as outlined in Hanaoka et al. (1979) and by SDS-PAGE of the fimbrial protein samples and silver staining (Merril et al., 1981). The presence of fibrils was determined by negative staining the electroeluted protein samples with ammonium molybdate, as outlined in the electron microscopy section.

Sephadex G-100 Fractionation of Fimbrial Proteins

Protein samples of *C. cinereus* PR2301 were eluted under non-

denaturing conditions with TEPI buffer from a 0.5 X 8 cm Sephadex G-100 column. Following elution of the void volume, 0.1 mL fractions were collected. Fractions containing proteins were determined by their absorbance at 280 nm using a Beckman DU 50 spectrophotometer. These fractions were concentrated by freeze drying and were suspended in a minimal volume of distilled water. The protein fractions were separated on an 11% SDS-polyacrylamide gel and then analyzed with the immunoblotting procedure.

Electron Microscopic Techniques

Shadow Casting

Oidia of *C. cinereus* were isolated from cultures 5 to 6 days old, grown on agar petri plates of CCM at 37° C. Approximately 5 ml of sterile distilled water was placed on each plate. The surface of the mycelium containing aerial hyphae was rubbed with a sterile glass rod to suspend the oidia in the distilled water. Hyphae of *S. commune* were isolated from cultures grown on agar petri plates of SCM at 22° C. Surface sections of the mycelia were suspended in sterile distilled water and macerated with a microhomogenizer. Both the oidial and hyphal samples were concentrated by centrifugation for 10 minutes at setting 6 in a clinical centrifuge. Samples were vigorously shaken in 20% acetone for approximately 15 seconds. These samples were centrifuged as before and the pellet was washed three times in distilled water. A drop of each suspension was applied to a 0.25% formvar coated and carbon reinforced 300 mesh copper grid. Cells were allowed to settle onto the surface of the grid for

approximately 1 minute. At this time, all but a small amount of the drop of the cell suspension was removed with a sliver of filter paper. It was important to remove the drop gradually from the grid to ensure that the cells on the grid were not pulled off. The grids were dried, mounted and secured into grid holders with a small amount of glue. The grid holders were placed within a Varian vacuum evaporator where gold was evaporated onto the surface of the grids at an angle of 20°. The grids were removed from the holders and viewed with a Philips 300 electron microscope.

Negative Staining

Drops of cell suspensions (as outlined in the shadow casting section) and protein preparations (as outlined in the electroelution section) were applied to grids in the same manner as outlined in the shadow casting section. However, before the grids dried, a drop of 3% ammonium molybdate (pH 7.5) was applied. The grids were stained for 30 to 60 seconds. At this time, all but a small amount of the stain was removed with a sliver of filter paper. After the grids had air dried, they were examined with a Philips 300 EM.

Grid Preparation

Formvar (Marivac) was gradually dissolved in ethylene dichloride to make a 0.25% formvar solution. Microscopic slides cleaned with 95% ethanol were dipped into the formvar solution and dried vertically in a dessicator. The formvar coated slides were scored with a razor blade to

allow the formvar to separate from the slide. The ends of the slides were then gradually submerged into a glass bowl of distilled water, previously swept with a glass rod to remove dust particles. Once the end of the formvar started to pull away from the slides, they were submersed until the formvar sheets had floated away from the slides on the surface of the water. Grids were placed carefully (shiny or smooth side down) on top of the formvar. The grids were then tapped gently with the forceps to produce a good contact between the grid and the formvar. The edge of a piece of stiff file card paper, cut to a size slightly larger than the formvar sheet, was placed against the far edge of the formvar sheet. The card was submerged at an obtuse angle, pulling the formvar with it. This produced a sandwich consisting of file card-grids-formvar. The sandwich was flipped over underwater and then resurfaced. After drying, carbon was evaporated onto the surface of the formvar using a vacuum evaporator (Varian). The prepared grids were stored in a dessicator at room temperature.

Inhibition of Conjugation with Antisera

Late log phase liquid CM cultures of *U. violacea* 1T10cb2 and 2716 cells were harvested by centrifugation in a clinical centrifuge at setting 6 for 5 minutes. The cell pellet was gently suspended in a small volume of sterile distilled water. Cells were washed three times in this manner. The final cell pellet was suspended in a small volume of sterile phosphate buffered saline (PBS) (pH 7.2). The concentrations of the two cell suspensions were determined using a hemocytometer and $1x10^6$ cells of each mating type was added to microcentrifuge tubes. Antiserum (AU or NS) was added to

individual tubes of cell suspensions in 0, 5, 12.5, 25 or 37.5 μL volumes. In addition, AU (12.5 μL) was preincubated with isolated *U. violacea* fimbrial protein and added to another cell suspension in a separate tube. All of the suspensions were then brought to 100 μL with PBS. Three replicates were performed for each of the above set-ups. All of the replicates were incubated overnight at 15° C and the next day, levels of conjugation were determined by phase contrast microscopy. Cells undergoing conjugation were identified by observing conjugation tubes between cells or by cells which remained in close approximation with one another even when moving. Two hundred randomly selected cells were scored from each replicate.

Results

Fimbriae on *Coprinus cinereus* and *Schizophyllum commune*

(I) Electron microscopic studies

The presence of fimbriae on *C. cinereus* and *S. commune* was determined directly with the use of negative staining and shadow casting. The fimbriae of *C. cinereus* were observed to be 0.5 to 20 μm in length. The lengths were determined from the electron micrographs in figures 1 and 2 and from direct observations under the electron microscope. The fibrils appeared to be flexuous and they did not taper. Furthermore, the fimbriae were very abundant on oidia. However, relatively few fimbriae were observed on the hyphal surfaces (data not shown). All of the electron micrographs of *C. cinereus* fimbriae were prepared from oidial samples. In figures 1 and 2, the fimbriae appear to be branched, but examination of negatively stained cells (Figure 3) indicated that this was not the case. In shadow cast preparations it is common to see fibrils coil around one another and then separate along the length of the fibril. This is probably an artifact that gives the impression that they branch. In negatively stained preparations, the addition of the stain apparently either separates the fibrils or maintains their separation. From negatively stained preparations, the diameter of the fibrils was estimated to be 5 nm. In addition, in figure 3 a repeating structure is apparent on the fibrils. This may indicate that these fibrils are helical in nature.

Figure 1. Shadow cast of *C. cinereus* oidium.

An electron micrograph of a *C. cinereus* oidium shadow cast with gold is shown. The fimbriae in this figure appear to be branched, but this is probably the result of coiling. The fimbriae in this electron micrograph were up to 20 μm in length. Magnification 23 000X.

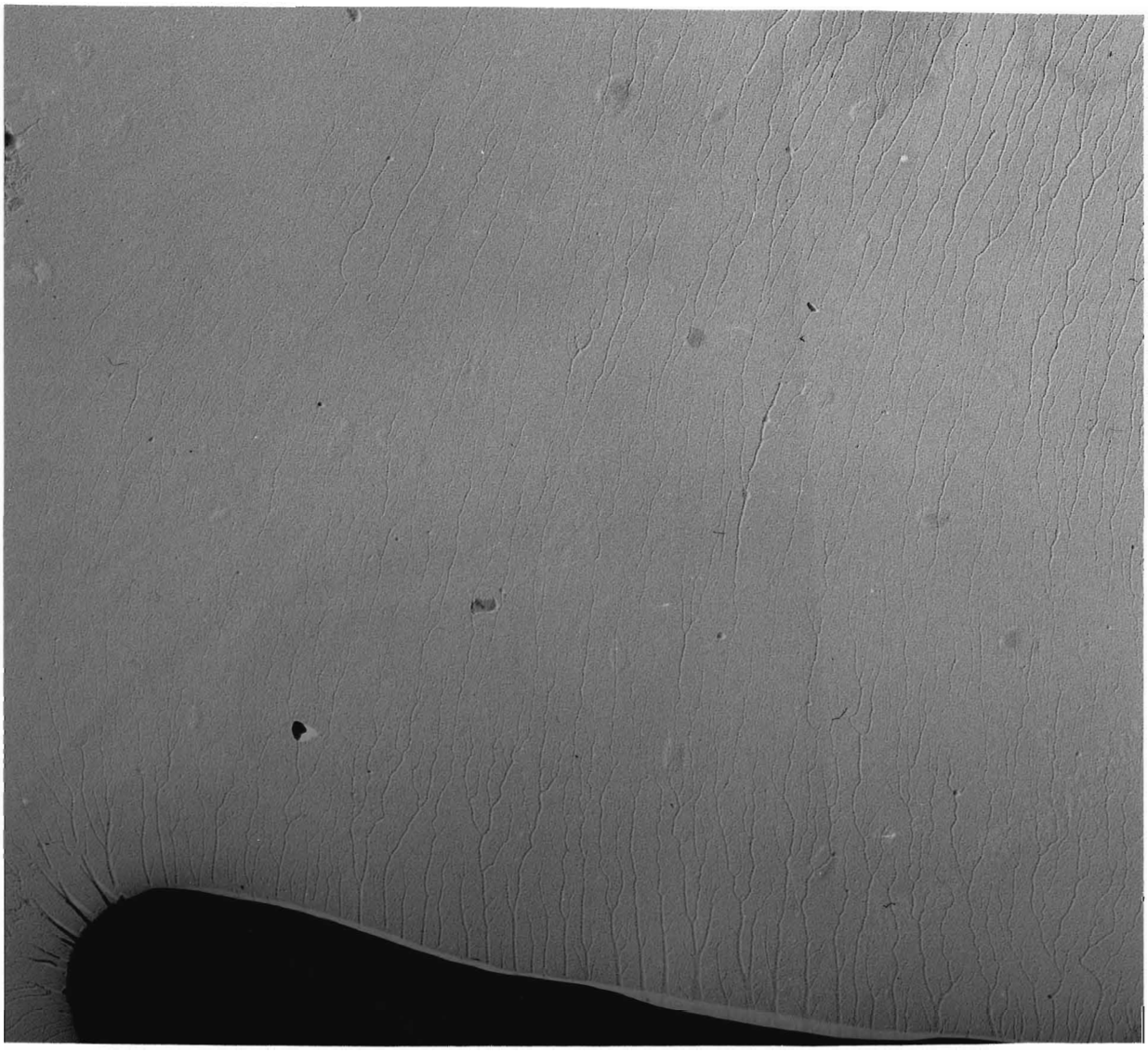
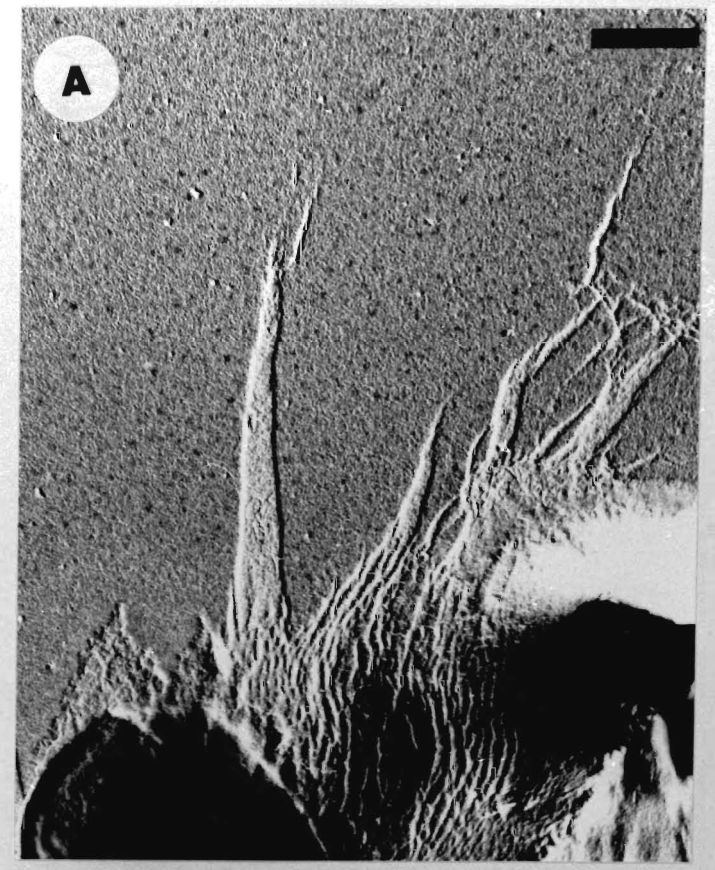


Figure 2. Shadow casts of *C. cinereus* oidia.

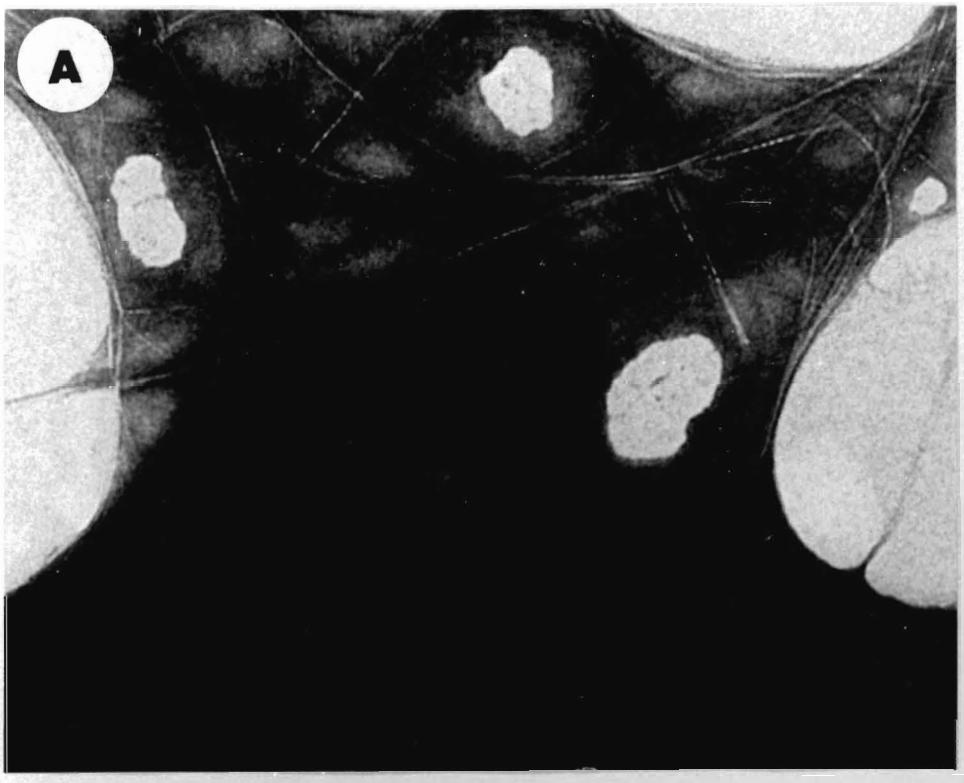
These electron micrographs (A and B) exemplify the coiling of fimbriae on the surfaces of oidia. Bar represents 1 μm.

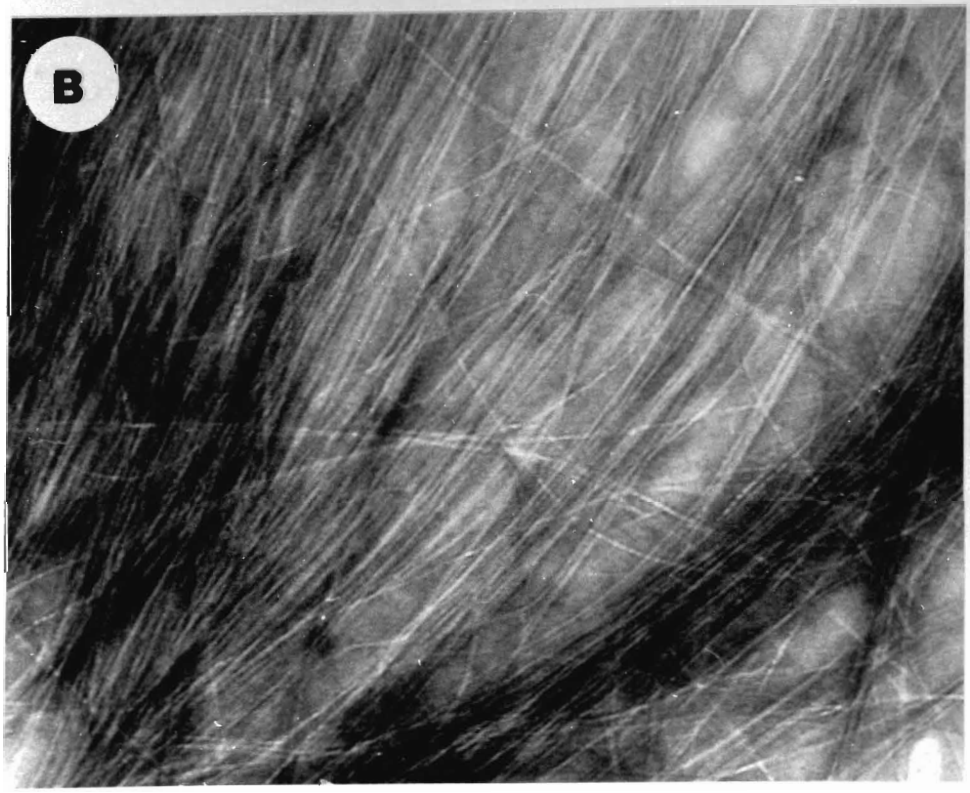

A


B

Figure 3. Negative stains of *C. cinereus* oidia.

Oidia negatively stained with ammonium molybdate indicate that the fibrils are not branched. The diameter of the fibrils were estimated to be 5 nm from these micrographs. In some of the fibrils, a repeating structure can be seen, possibly indicating that the fibrils are helicies. Magnification 100 000X.


A


B

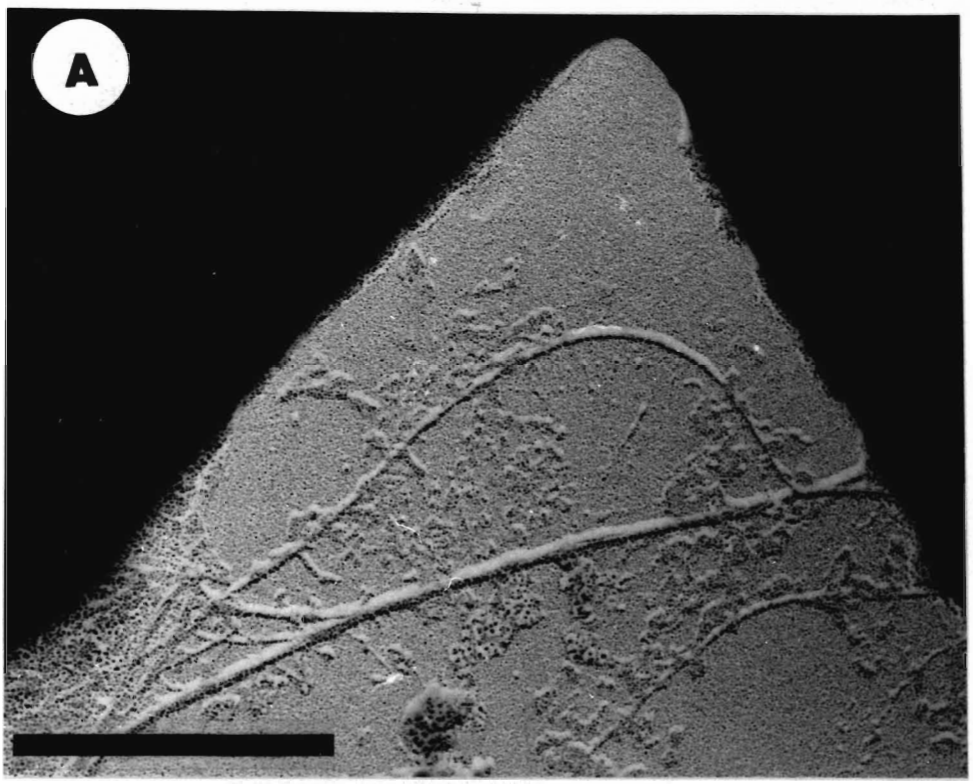
Unlike *C. cinereus* , *S. commune* does not have a vegetative unicellular stage. When preparing the hyphae of *S. commune* , it was necessary to be very careful not to disturb them on the grids. The hyphae had a tendency to bunch together and to pull off of the grid during the removal of the excess cell suspension or stain. The fimbriae of *S. commune* were generally 0.5 to 2 μm in length (Figure 4). These lengths were determined from figure 4 and during direct examination of the fibrils using the electron microscope. As in *Coprinus* , they appear to be flexuous and they do not taper. In the hyphae observed, fimbriae were sparsely distributed over the entire cell surface (figure 4). However, higher densities of the fibrils were observed in the older sections of the mycelium, especially on small side growths on the hyphae (Figure 4). Again, fimbriae identified by shadow casting appear to be branched, but negatively stained preparations (Figure 5) indicate that this is not the case. From the micrographs of negatively stained preparations, the diameter of the fibrils was estimated to be approximately 5 nm.

(II) Immunoblot Analysis

C. cinereus and *S. commune* proteins homologous to the fimbrial protein of *U. violacea* were identified in immunoblots with AU (Figure 6). Two proteins of the *C. cinereus* mycelial sample cross-reacted with AU, a minor band at 39 +/-3 kd and a major band at 37 +/-3 kd. The errors were calculated from four separate isolations and immunoblot analyses. AU also bound to the 37 kd protein in the oidial sample. Two proteins, one 89 kd and the other 92 kd, were bound very weakly by AU in the *S.*

Figure 4. Shadow casts of *S. commune* .

Electron micrographs of hyphae shadow cast with gold indicate the presence of fimbriae on *S. commune* . (A) Between two overlapping hyphae, coiled fimbriae can be seen. (B) In the older sections of the mycelium, these side extensions of the hyphae are commonly found to be fimbriated. Bars represent 1 μm.


A


B

Figure 5. Negative stain of *S. commune* .

An electron micrograph stained with ammoniummolybdate indicates that the fibrils are not branched. The diameter of the fibrils was estimated to be 5 nm. Magnification 100 000X.

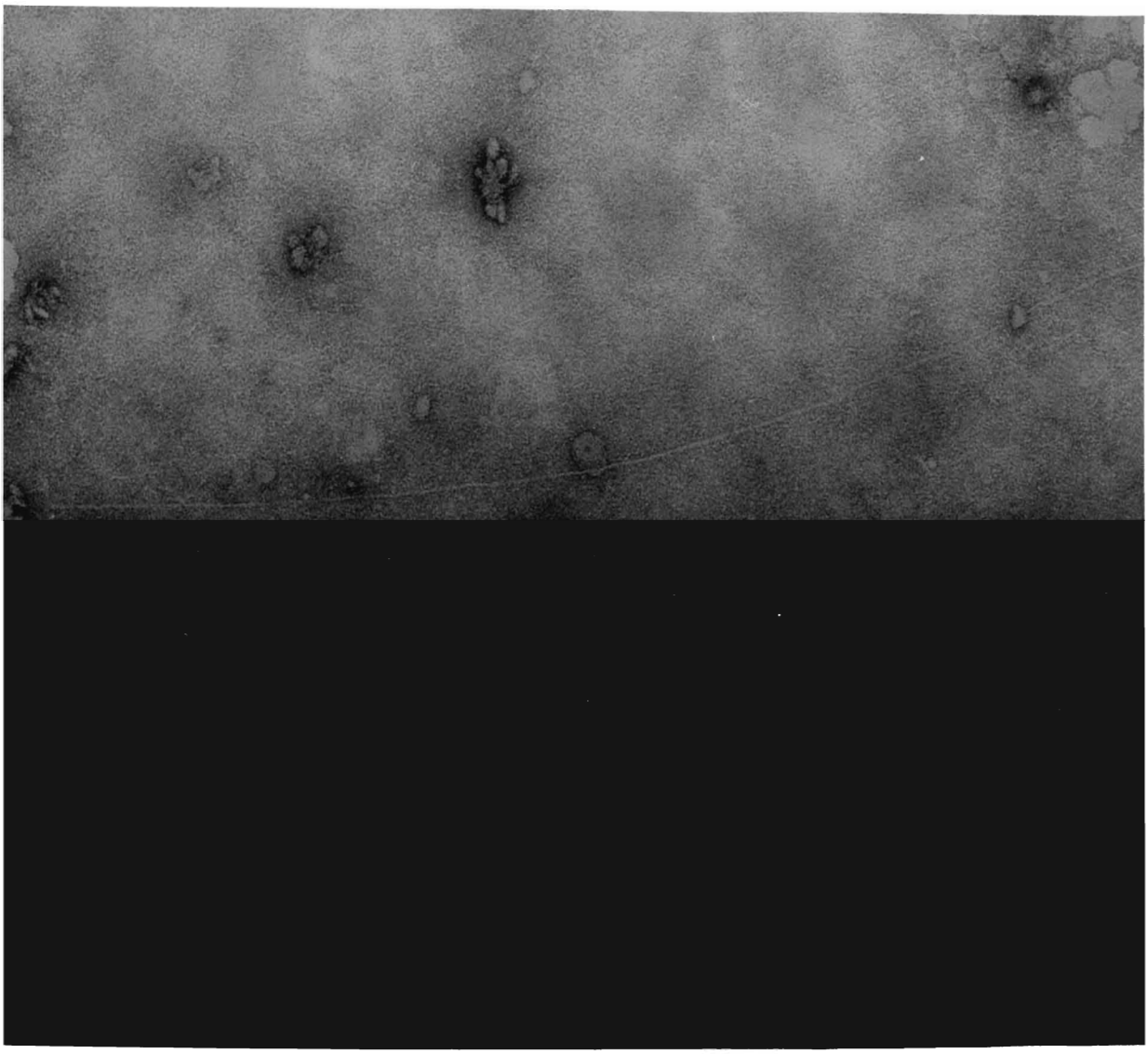
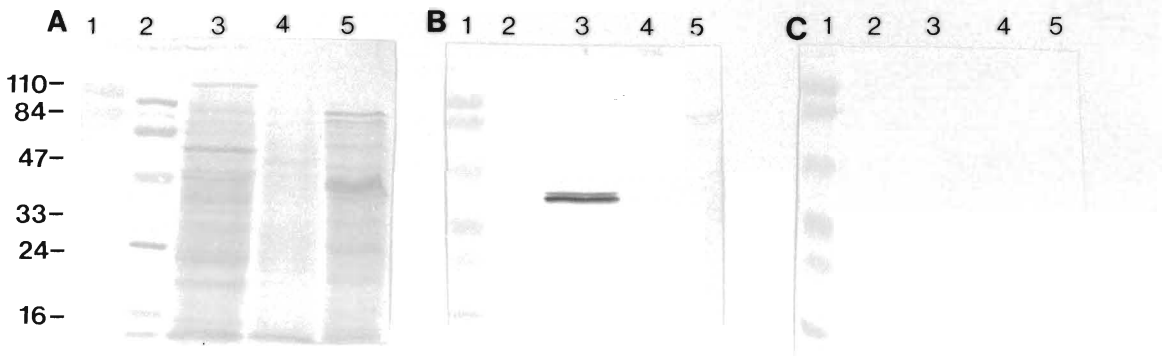


Figure 6. Immunoblot of the total proteins of *C. cinereus* and *S. commune* .

(A) Amido black stained nitrocellulose membrane showing the proteins transferred from the SDS-PAGE gel. Numbers to the left of the blot indicate the molecular weight of the prestained standards (lane 1) in kilodaltons. (B) Immunoblot bound with antibodies raised against the fimbrial proteins of *U. violacea* (AU). This shows that two *C. cinereus* proteins (37 and 39 kd) cross-react with AU (lane 3). AU also recognizes these proteins from oidia (lane 4). The intensity difference is due to a ten fold difference in the amount of protein present between the samples. AU bound weakly to two *S. commune* proteins (89 and 92 kd) (lane 5). The amount of protein loaded in the *C. cinereus* and *S. commune* lanes was the same. (C) Controls employed were incubation of the blots with pre-immune serum (NS), 2° without 1° antibodies and preincubation of AU with isolated *U. violacea* fimbrial protein. No binding was observed with any of these treatments. The immunoblot incubated with NS is shown.

1. Prestained low molecular weight markers.
2. Low molecular weight markers.
3. *C. cinereus* mycelial protein.
4. *C. cinereus* oidial protein.
5. *S. commune* mycelial protein.


A
1
2
3
4
5
110–
84–
47–
33–
24–
16–
B
1
2
3
4
5
C
1
2
3
4
5

commune mycelial sample. The total protein separated on the gels was the same for the mycelial samples of *C. cinereus* and *S. commune* . Thus, AU recognized *S. commune* fimbrial proteins very weakly. Repeated analyses yielded similar results. Consequently, no further analyses of *S. commune* fimbrial proteins were undertaken.

Several control experiments confirmed the specificity of AU recognition of the putative fimbrial proteins of *C. cinereus, S. commune* and *U. violacea* . No reaction was observed when: 1) the AU antibodies were preincubated with isolated *U. violacea* fimbrial protein, 2) preimmune serum (NS) was used instead of AU or 3) the 1^{o} antibody was omitted. The NS control is shown in figure 6C.

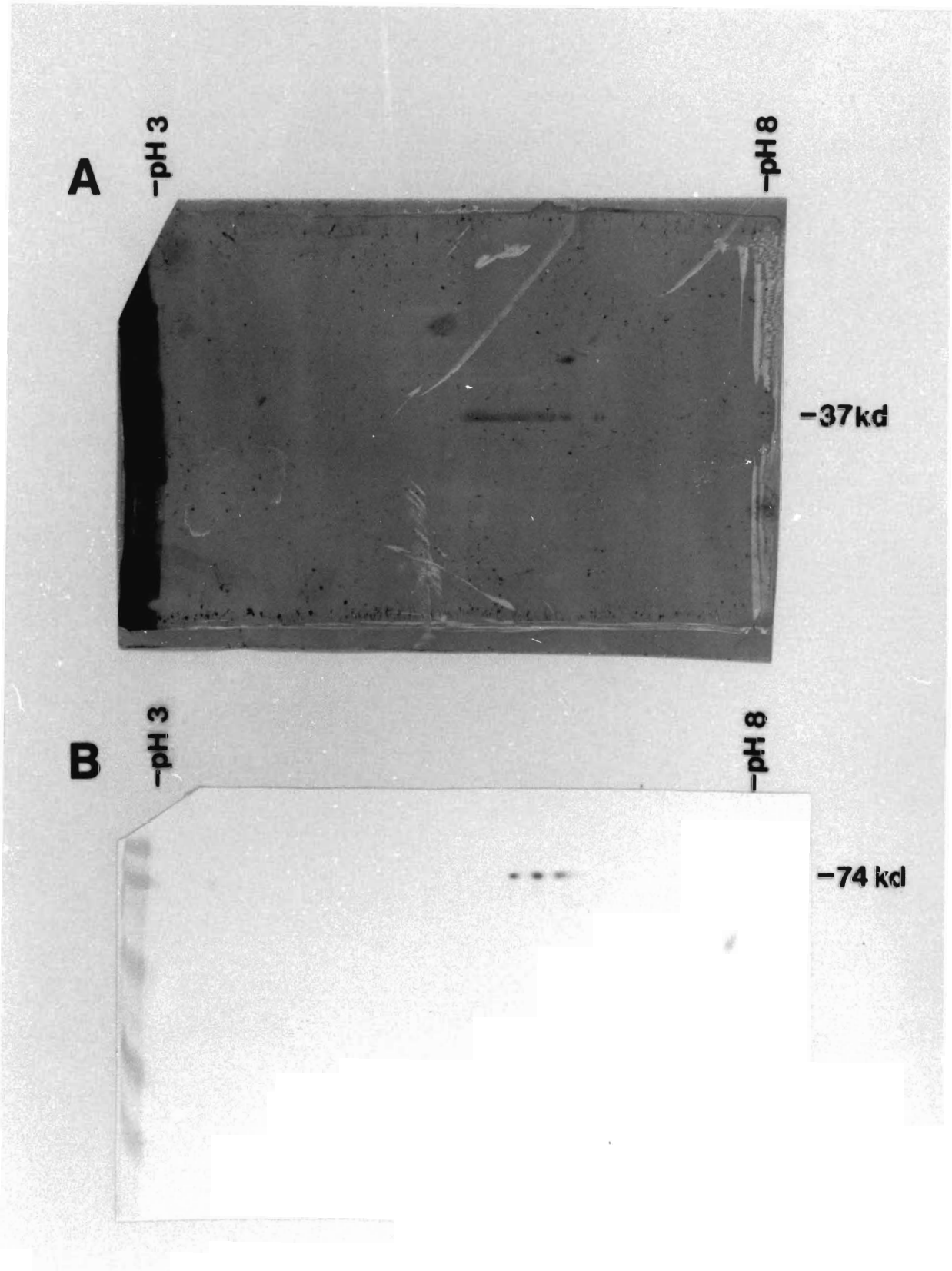
Protein samples isolated from *C. cinereus* mycelium were also separated with 2-D gel electrophoresis. An immunoblot analysis of such a gel is shown in figure 7A. The proteins visualized by AU were all approximately 37 kd in molecular weight. Their isoelectric points were as follows: pH 6.10-7.20, pH 7.37, pH 7.59 and pH 7.63. The high background was produced by nonspecific binding of alkaline phosphatase-streptavidin conjugate to the nitrocellulose. The streptavidin was used to bind to a set of biotinylated low molecular weight standards in this particular esperiment.

Electroelution of *Coprinus* Fimbrial Protein

Even though AU antiserum cross-reacted with proteins of *C. cinereus* , these proteins may not be the fimbrial proteins of *C. cinereus* . To prove that they were fimbrial proteins, it was necessary to isolate these proteins

Figure 7. 2-D immunoblots of *C. cinereus* and *U. violacea* proteins.

An immunoblot of *Coprinus cinereus* mycelial protein (A) and *Ustilago violacea* fimbrial proteins (B) separated by 2-D gel electrophoresis. (A) The 37 kd *C. cinereus* mycelial proteins bound by AU were pH 6.1-7.2, pH 7.37, pH 7.59 and pH 7.63. (B) The 74 kd fimbrial proteins of *U. violacea* bound by AU were pH 6.76, pH 7.07 and pH 7.25.


A
pH 3
pH 8
-37kd
B
pH 3
pH 8
-74 kd

and determine if they could form fibrils the same diameter as the fimbriae observed on the oidia of *C. cinereus* . The putative fimbrial proteins of *C. cinereus* were separated on SDS-polyacrylamide gels and then the proteins from the gels were electroeluted. The success of the electroelution procedure was determined by separating the eluted proteins on an SDS-polyacrylamide gel. Furthermore, it was possible to electroelute cytochrome C (pI 9.6). This indicated that proteins with isoelectric points less than 9.6 (pI 0-9.6) could be eluted with the buffer system employed. Most proteins have pIs within this range. When the eluted *C. cinereus* proteins were applied to grids and negatively stained, fibrils were observed under the electron microscope (figure 8). The diameter of these fibrils was determined to be approximately 5 nm.

Sephadex G-100 Separation of *Coprinus* Fimbrial Proteins

The *C. cinereus* proteins bound by AU were found to be able to form fibrils the same diameter as *C. cinereus* fimbriae. Since the 37 and 39 kd proteins could not be individually electroeluted due to their similar size, it was of interest to see if both of these proteins were capable of forming fibrils. An immunoblot analysis of *C. cinereus* mycelial proteins eluted from a Sephadex G-100 column is shown in figure 9. The 37 kd protein was eluted from the column immediately after the void volume (fraction 1) and was almost completely eluted by fraction 10. However, the 39 kd protein was not eluted from the column until fraction 6 and was still being eluted in large amounts in fraction 10.

Figure 8. Electroelution of fibrillar protein from *C. cinereus* .

An electron micrograph of negative stained proteins electroeluted from *C. cinereus* total protein indicates the presence of multimeric proteins in this sample. The diameter of the fibrils is 5 nm. Magnification 100 000X.

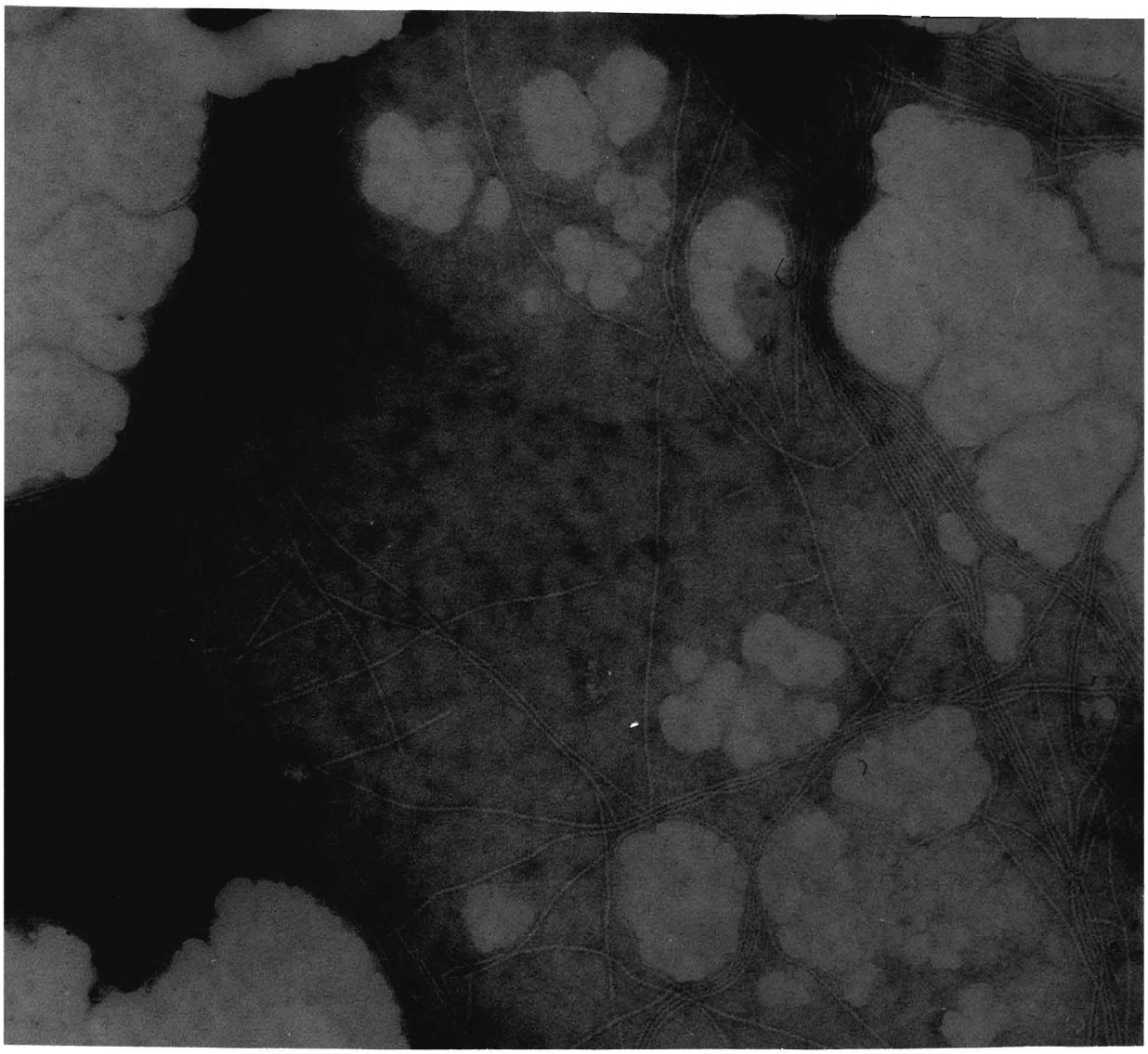
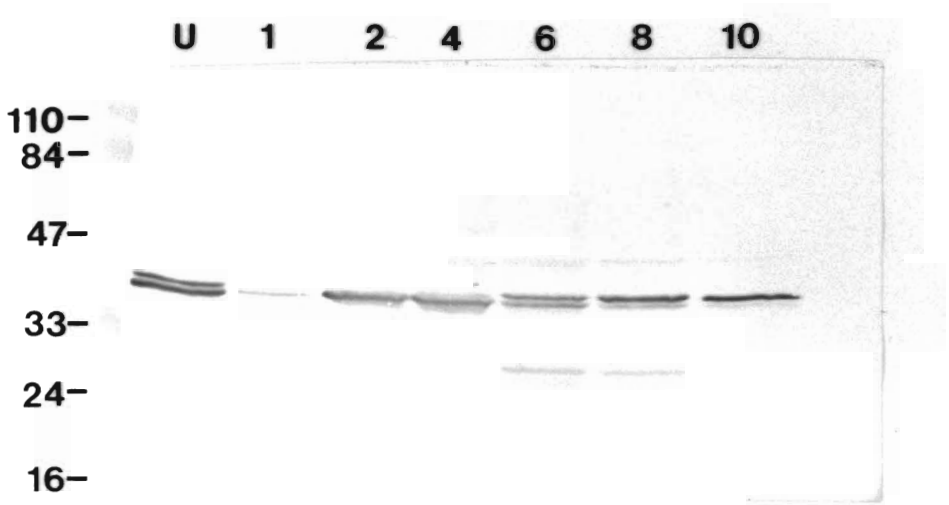


Figure 9. Determination of the multimeric nature of the proteins recognized by AU.

Immunoblot of *C. cinereus* total protein (lane U) and the fractions collected after this sample was eluted through a Sephadex G-100 column under non-denaturing conditions. The 37 kd protein is found in the first fraction, to a larger extent in fractions 2 and 4, and is almost completely eluted by fraction 10. However, the larger 39 kd protein is not eluted until fraction 6 and is found predominately in the last fractions. Prestained molecular weight markers are present on the left of the figure and the molecular weights shown are in kilodaltons.

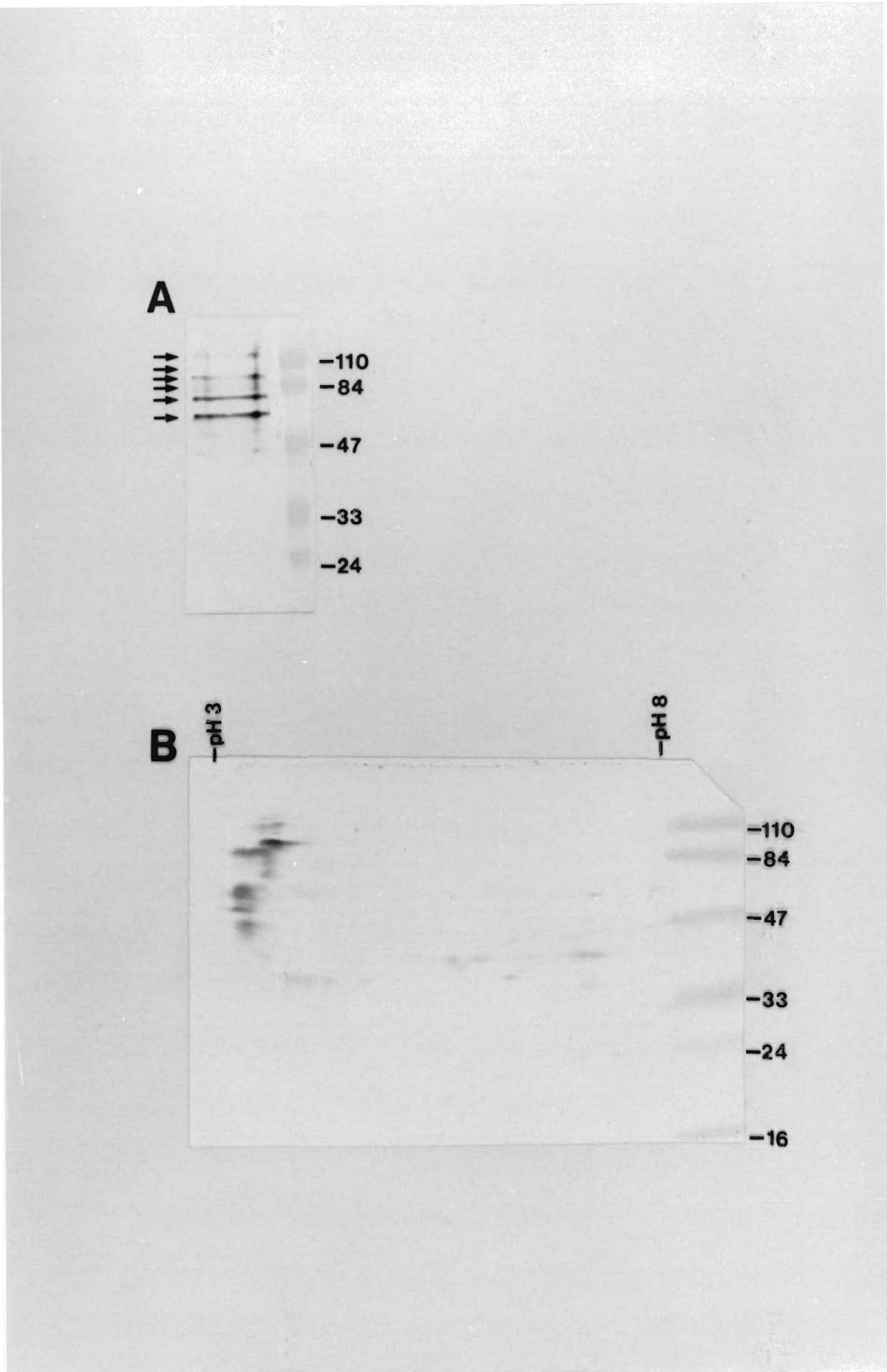

U
1
2
4
6
8
10
110–
84–
47–
33–
24–
16–

Immunoblot Analysis of *Ustilago* Proteins

Immunoblot analysis of *Ustilago* proteins had not been performed prior to the start of this thesis work. In order to determine the specificity of the interaction between AU and the proteins of *S. commune* and *C. cinereus* , it was necessary to perform this analysis on the proteins of *U. violacea* . Six different major proteins are recognized by the antiserum against *Ustilago* fimbrial protein (AU) (Figure 10). The molecular weights (MW) of these proteins were 117 +/-21, 93 +/-11, 85 +/-7, 80 +/-9, 74 +/-5 and 61 +/-3 kd. The standard errors ($X^{+}sX{\cdot}t$) were calculated from four separate immunoblot analyses of different protein samples. Presumably, the 74 kD protein corresponds to the 74 kd fimbrial protein (Gardiner and Day, 1985). *Ustilago* total protein was separated by two-dimensional gel electrophoresis and this procedure produced a consistent pattern of protein separation in both dimensions. Figure 7B shows a 2-D gel of *U. violacea* 2716 protein assayed with the immunoblot procedure. In general, the proteins visualized were acidic. The predominant proteins bound by AU were 112 kD (pH 3.7-4.1), 95kD (pH 3.7-4.1), 87 kd (pH 3.2-3.8), 63 kd (pH 3.2-3.6), 54 kd (pH 3.22-3.6) and 47 kd (pH 3.2-3.6). The molecular weights and isoelectric points were calculated from the immunoblot in figure 10. The range in their isoelectric points represents the spot-lengths on the immunoblot. When concentrated samples of isolated *Ustilago violacea* 2716 fimbrial protein were separated on a 2-D gel and stained with Coomassie blue, three 74 kd spots were observed (data not shown). All

Figure 10. Immunoblots of *Ustilago violacea* proteins.

(A) *U. violacea* proteins were separated by SDS-PAGE and immunoblotted with AU. The six proteins bound by AU were approximately 117, 93, 85, 80, 74 and 61 kd in molecular weight. (B) *U. violacea* proteins were separated by 2-D gel electrophoresis and immunoblotted with AU. The proteins bound by AU were approximately 112 kd (pH 3.7-4.1), 95 kd (pH 3.7-4.1), 87 kd (pH 3.2-3.8), 63 kd (pH 3.2-3.6), 54 kd (pH 3.22-3.6) and 47 kd (pH 3.2-3.6). Prestained molecular weight markers and their sizes in kilodaltons are present on the right side of both figures.


A
110
84
47
33
24
B
pH 3
pH 8
110
84
47
33
24
16

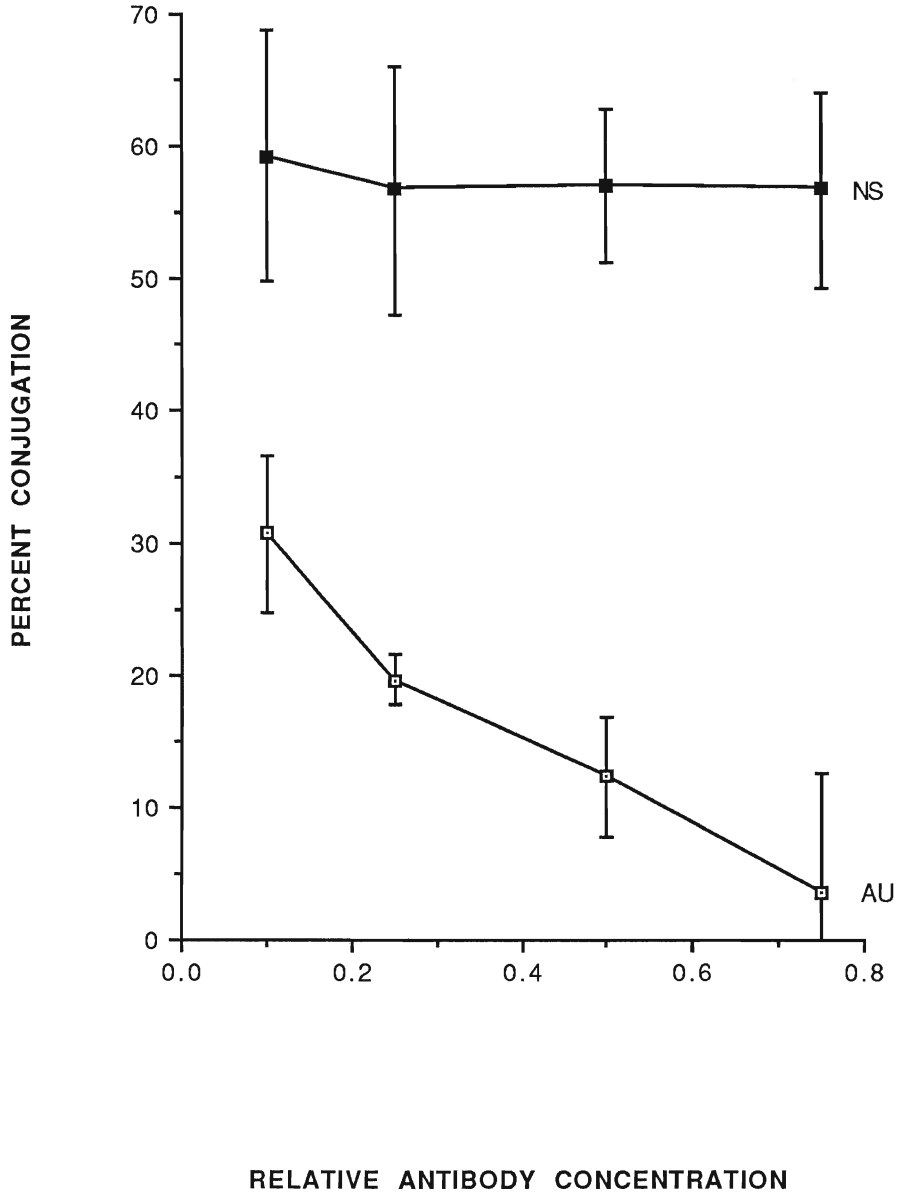
three of these proteins were recognized by AU (Figure 7B). The isoelectric points of these proteins were approximately pH 7.25, 7.07 and 6.76.

Inhibition of Conjugation in *Ustilago*

The effects of the presence of AU on conjugation in *U. violacea* is shown in figure 11. As the relative concentration of AU was increased, there was a significant ($U_{0.01(2)3,3}$) decrease in the percentage of cells mating according to the nonparametric Mann-Whitney test. The concentration of the antibodies in serum is difficult to determine due to contamination by other components. Thus, when comparing the concentrations of AU and NS antibodies, the protein concentrations were determined with the Bradford method and the more concentrated serum was diluted to the same concentration as the less concentrated serum. It was then assumed that antibodies in the two sera were approximately the same concentration. When the relative concentration of NS was increased, there was no significant decrease in the percentage of conjugation ($U_{0.10(2)3,3}$). Furthermore, the levels of conjugation in each of the NS serum concentrations used were not different from control (no antiserum) levels of conjugation ($U_{0.10(2)3,3}$) which were assayed in the presence of PBS. However, the percentage of conjugation in each of the AU serum concentrations were significantly lower than both their respective NS serum concentrations and the PBS no antiserum control ($U_{0.10(2)3,3}$). In addition, when AU antibodies were preincubated with isolated *Ustilago* fimbrial protein, the level of conjugation was significantly higher then with AU antibodies not preincubated with fimbrial protein ($U_{0.10(2)3,3}$). For the

Figure 11. Inhibition of conjugation in *U. violacea* .

This graph shows the inhibition of conjugation between *U. violacea* strains 1T10cb2 (a1) and 2716 (a2) with AU (0) in comparison to the same strains incubated with NS (o). Control levels of conjugation with PBS were 56.7 +/- 4.5%. AU preincubated with isolated fimbrial protein (47.5 +/- 2%) was significantly higher than with just AU alone (13.0 +/- 2.5%). Error bars represent confidence intervals (95%).


70
60
50
40
30
20
10
0
0.0
0.2
0.4
0.6
0.8
NS
AU
PERCENT CONJUGATION
RELATIVE ANTIBODY CONCENTRATION

preincubation experiment, the antibodies used were isolated with a protein-A column (Schleicher and Schuell). Thus, the antibody solutions were comprised entirely of IgG. This procedure allowed a more precise determination of the amount of fimbrial protein that would have to be added to the AU antibodies. In this instance, fimbrial protein was incubated with AU IgG in a 2:1 mole ratio.

Discussion

Until this study, fimbriae on the surfaces of *C. cinereus* and *S. commune* had not been observed in any manner. The dimensions of fimbriae on these two species are similar to those of other fungi. It is not surprising that these fungi are fimbriated. In fact, most fungi examined to date have been shown to be fimbriated (Gardiner, 1981, 1982). This suggests that fimbriae perform an important and conserved function in these fungi.

The distribution of fimbriae on *C. cinereus* is not uniform. The unicellular asexual propagule of *C. cinereus* , the oidium, has been found to be more fimbriated than its hyphal stage. Jurand and Kemp (1971) have shown that oidia of the fungus *Psathyrella coprophila* , another member of the Coprinaceae, have numerous surface fibrils. These fibrils appeared to be very similar to the fimbriae seen in this study. This distribution of fibrils is similar to the fimbriation of *U. violacea* ; in this fungus, the fimbriae present on sporidia are generally longer and more plentiful than the fimbriae on hyphae (Gardiner, 1985). What would be the reason for the increased expression of fimbriae on the unicellular stage compared to the mycelial stage? One can speculate that these fibrils perform an important role for these unicellular propagules. Increased fimbrial production may be necessary for a function expressed in the unicellular stage. Although this function may also be performed by the mycelium, it may be more critical to the survival of the unicellular fungus. A mycelium is a large network of intertwining hyphae. The large surface area and interconnections between cells allow it to survive better. All of the individual cells contribute to the community of cells. In contrast, an

oidium lacks this network and the corresponding advantages. It is possible that fimbriae may be a contributing factor to the survival of the odium. These fibrils may permit the fungus to explore areas up to 10 µm from its cell surface. Like the fimbriae of bacteria, they may be involved in adhesion (Brinton, 1965). Once fimbrial contact is made with a suitable host, nutrient source or compatible mating strain, the fimbriae may bind to a specific surface macromolecule. This would keep the cell in close proximity to the bound substrate. Any interactions between the fungus and the bound substrate would therefore be performed more easily. Fungi lacking these appendages would have a decreased area of surveillance; thus, their viability would be diminished. In *Coprinus* , this fimbrial contact may be responsible for the induction of hyphal growth towards oidia. Hyphal growth towards an oidium occurs once the hyphae has received a "stimulus" from the oidium (Bistis, 1970; Kemp, 1970). Possibly this "stimulus" is produced by fimbrial contact between oidium and hypha. In nature, *Coprinus* oidia are produced on the surface of dung. In order for contact between an oidium and a compatible monokaryon to take place, there must be a means of dispersal present. One way in which oidia are dispersed is by means insects (Brodie, 1931). Fimbriae may facilitate this transport function by attaching to the insect. However, the role of fimbriae may also be structural in nature. Fimbriae may hypothetically serve as suspensors. Fimbriated bacteria form pellicles on the surfaces of static liquid medium. This phenomenon is thought to increase access of the bacterium to an aerobic environment (Ottow, 1975). A similar mechanism may be operative in *Coprinus* and *Ustilago* . In addition, the ability of an oidium to float may aid in its dispersal. The ability of conidia, the vegetative spores of ascomycetes, to float aid in their dispersal (Rees,

1980). Thus, fimbriated cells may be carried further than cells without fimbriae.

An alternative explanation for the differences observed between the fimbriation of single cells and of hyphae is that the differences are artifactual. Single cells are easier to work with than hyphae. Fewer and less rigorous manipulations of yeast-like cells are required for electron microscopic examination. It is possible that, the fimbriae of the hyphal samples were lost or swept along the hyphal surface during their preparation. This would effectively negate their presence. Previous observations argue against this hypothisis. First and foremost, the fimbriae of *Ustilago violacea* and other fungi are very stable (Gardiner and Day, 1985). They survive several harsh chemical and enzymatic treatments. Second, fimbriae of *U. violacea* regenerate quickly (Poon and Day, 1974, 1975). If fimbriae were sheared from hyphae during experimental manipulations, they should have had time to partially regenerate. Thus, the most probable conclusion is that the differences in fimbriation of single cells such as oidia or sporidia and hyphae are real.

There was also an unequal distribution of fimbriae on *S . commune* . The side growths found in the older sections of the mycelia of *S. commune* were more fimbriated than other sections. This is particularly interesting, since the incidence of hyphal anastomosis is also higher in the older sections of the mycelium (Ward, 1888; Buller, 1933). In *S. commune* , hyphal anastomosis may occur tip to side (Raudaskoski, 1973) or tip to tip (Buller, 1933). In *Achlya ambisexualis* and *Mucor* spp. the growth of hyphae towards a chemical signal is well established (reviewed in Horgen, 1981; Trinci, 1984). In *S. commune* , it is difficult to imagine that a chemical signal could create a tip to tip fusion event within a complex

mycelium. There would be an enormous number of competing signals which should make directed hyphal growth unlikely. Similar to the postulated role of fimbriae during conjugation in *U. violacea* (Day and Poon, 1975), fimbriae may establish preliminary contacts between anastomosing hyphae. Theoretically, hyphal growth would proceed along these fimbrial routes. This could explain the accuracy of tip to tip fusions. The clusters of fimbriae on the tips of hyphal side branches in *S . commune* may provide this contact function.

Partial identification of the fimbrial proteins of *S. commune* and *C. cinereus* was attempted with immunoblot procedures with the antiserum against *U. violacea* fimbrial protein. The proteins of *S. commune* were bound by AU very weakly. It was not surprising that AU did not cross-react with the fimbrial proteins of *S. commune* . AU does not bind to the surface antigens of several fungi (Gardiner et al., 1981; 1982), but does to certain algal species (Day et al., 1986b). There is no discernable pattern to the conservation of fimbrial antigens except within the Ustinaginaceae. Thus, it is impossible to predict the cross-reactivity of AU based on phylogeny. The inability to detect any *S. commune* proteins that cross-reacted strongly with AU made it difficult to characterize the fimbrial proteins of this fungus. As a result, the fimbrial proteins of *S. commune* could not be further characterized in this study. Because of the intriguing possibilities mentioned above, the fimbrial proteins of *S. commune* should be further characterized in the future using alternative techniques. One approach, would be to test the cross-reactivity of the fimbrial proteins of *S. commune* with other antisera such as AR. Alternatively, once the fimbrial gene(s) of *U. violacea* and other fungi have been isolated and cloned, then the fimbrial gene(s) of *S. commune* could be identified. The success of this

approach would depend on the presence of DNA sequences which are conserved between genes from different species or a shared linkage to other loci. With the gene, the protein could be initially characterized based on the DNA sequence alone.

A prominent 37 kd and a minor 39 kd protein of *C. cinereus* reacted strongly with AU. This result indicated that these proteins share the same epitopes with the fimbrial proteins of *U. violacea* . These proteins were likely to be the fimbrial proteins of *C. cinereus* , but confirmation was necessary. As indicated with *S. commune* , AU does not cross react with fimbrial protein from every fungal species. In addition, although it has not been proven, it is possible that AU may recognize non-fimbrial proteins which have epitopes in common with the fimbrial protein. Therefore, it was necessary to combine both immunoblotting and EM observations to allow definitive proof of association between the two *Coprinus* proteins and fimbriae. When these proteins were electroeluted from SDS-polyacrylamide gels, they were able to form fibrils. These fibrils were the same diameter as the oidial fimbriae. From these observations, the 37 and/or 39 kd proteins are the fimbrial proteins of *C. cinereus* . Since these proteins could be identified with AU unlike the *S. commune* proteins, they were further characterized.

Two-dimensional Western blots of the total mycelial protein of *C. cinereus* indicated that the 37 kd protein was in reality several proteins. The fimbrial proteins of *U. violacea* are also composed of multiple isoelectric forms (Gardiner et al, 1979; Castle et al., manuscript in preparation). In addition, the isoelectric points of the *U. violacea* and *C. cinereus* fimbrial proteins were approximately the same. Whether this association is fortuitous or a necessary aspect of fimbrial structure is

unknown. Further studies comparing fimbrial proteins from several species should resolve this uncertainty.

The 37 kd proteins appeared to be multimeric, since they were eluted from a Sephadex G-100 column immediately after the void volume. Thus, these proteins were capable of forming fibrils. All of this evidence strongly suggests that the 37 kd proteins are the fimbrial proteins of *C. cinereus* . This would be the smallest fimbrial protein reported for fungi. The size difference between *C. cinereus* and *U. violacea* fimbrial proteins may reflect functional differences for the fimbriae of these two species. In most bacteria, the adhesive ability of fimbriae is performed by a minor protein that is part of the fimbrial structure (Lindberg et al., 1984). Thus, the fimbriae of *C. cinereus* may be structurally similar to the fimbriae of bacteria. They may have minor components which perform functional roles apart from forming the ultrastructure. The larger fimbrial proteins of *U. violacea* and *R. rubra* may have domains which fulfill the roles of the hypothetical minor protein(s) of *C. cinereus* . Alternatively, *C. cinereus* fimbriae may lack functions associated with these other fungi. Sequence or peptide fragment analysis of fimbrial proteins from different fungal species may provide insight into functional domains of the protein. It will be interesting to see if other hymenomycetes also produce small fimbrial monomers, or whether it is isolated to *C. cinereus* .

Unlike the 37 kd proteins, the 39 kd protein was not multimeric. The 39 kd protein was eluted from a Sephadex G-100 column several fractions after the void volume. This protein, which shares an epitope(s) with the fimbrial proteins, may be of two different types: 1) a precursor to the mature fimbrial proteins or 2) a functionally unrelated protein which has homologous amino acid domains to the fimbrial proteins. In order for an

extracellular surface fibril to be assembled, the structural subunits must be exported from the cell. In *E. coli* , fimbrial proteins contain signal peptides which are required for their transport to the periplasmic space (Mooi and deGraaf, 1985). In eukaryotes, analogous transport signal peptide sequences are generally 15 to 30 amino acids in length (approximately 1800-3600 d) (Silhavy et al., 1983). The 39 kd protein could conceivably be a precursor for the 37 kd fimbrial protein. The signal peptide may inhibit fimbrial subunit binding. Phage tail proteins (King, 1980) and flaggelin subunits (Inno, 1977) are synthesized in a form that cannot spontaneously form fibrils. This ensures that polymerization only occurs at the correct site. Likewise, the signal peptide may ensure that fimbriae may only be formed once transported across the cell membrane. This would prevent the formation of any "free" unprocessed fimbrial proteins within the cytoplasm. However, it may be possible that the 39 kd protein is not a precursor to the fimbrial protein, but a distinct protein which shares homologous amino acid domains to the fimbrial proteins. In *E. coli* , fimbriae are anchored to the cell wall by a protein that is very similar to the structural fimbrial protein. This protein and the bacterial fimbrial protein probably share domains necessary for fimbrial subunit binding (Baga et al., 1987). Thus, in *C. cinereus* , another component of the fimbrial structure may be recognized by AU due to its homology to the structural 37 kd fimbrial proteins.

In *U. violacea* , six major proteins cross-reacted with AU on immunoblots of 1D gels. It was expected that the 74 kd fimbrial protein would be bound by AU, but the other five proteins were unexpected. Similar to the 39 kd protein of *C. cinereus* , these proteins may be precursors to the mature fimbrial proteins and/or distinct proteins which

have similar epitopes to the fimbrial protein. The fimbrial proteins of *U. violacea* are known to be phosphorylated (Day and Cummins, 1981) and glycosylated (Castle et al., manuscript in preparation). Thus, extensive processing of the nascent fimbrial polypeptide may occur in this organism.

In order to address the role of fimbriae in hyphal anastomosis and clamp connection formation, a suitable model system would be beneficial. Correlations between presence and function can be made, but these are only speculative. Due to the inability to recover stable fimbrial mutants in *U. violacea* , the determination of fimbrial function has been difficult. Thus, the possible functions that fimbriae might perform are based entirely upon correlations. In *U. violacea* , there is a strong correlation between the presence of fimbriae and the ability of mating sporidia to undergo conjugation (Day and Poon, 1975). In this study, AU antibodies were used to inhibit conjugation in *U. violacea* . It was not possible to completely block conjugation with AU, but it was inhibited approximately ten fold. However, with increasing concentrations of AU, there was a corresponding decreasing trend in the level of conjugation. Furthermore, these levels of conjugation were all significantly lower than the various controls. It may have been impossible to block conjugation completely. Fimbriae are thought to form preliminary links between cells of opposite mating type, inducing the cells to conjugate (Day and Cummins, 1974, 1975). If cells were already in close contact with one another prior to mating, then a fimbrial connection may be unnecessary for the induction of conjugation. Moreover, the density of fibrils would be larger between the two compatible cells. Thus, the probability of inhibiting fimbrial contact in this instance would be lower. This may explain the low level of conjugation observed in the presence of high AU concentrations. These

data provide strong evidence for the involvement of fimbriae in conjugation and pave the way for similar studies on other types of cell fusions in fungi.

In this study, fimbriae have been postulated to be involved in hyphal anastomosis and several functions involved in the successful propagation of vegetative spores and spermatia based on the distribution of their fimbriae. With this preliminary evidence, it would be interesting to test the involvement of fimbriae in these functions. In *U. violacea* , conjugation was inhibited significantly with AU. This is further evidence for the role of fimbriae in conjugation. Since the fimbriae of *C. cinereus* cross-reacted with AU, it would be particularly interesting to examine the effect of AU on the formation of clamp connections and hyphal anastomosis in this fungus. Part of the problem in addressing the function of fimbriae has been the inability to produce stable fimbrial mutants. Once the fimbrial gene is isolated, this problem should be overcome and the analysis of fimbrial function can proceed along more direct lines of evidence. In light of the proteins bound by AU in *U. violacea* and *C. cinereus* , it will also be interesting to determine the assembly and the exact posttranslational modifications that fimbriae undergo.

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